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GRADUATE COLLEGE

DISCOVERY, PURIFICATION AND CHARACTERIZATION OF ANTIFUNGAL PROTEINS AND OTHER DEFENSE-RELATED COMPOUNDS FROM TROPICAL PLANTS

A Dissertation

SUBMITTED TO THE GRADUATE FACULTY

In partial fulfillment of the requirements for the

degree of

Doctor of Philosophy

By

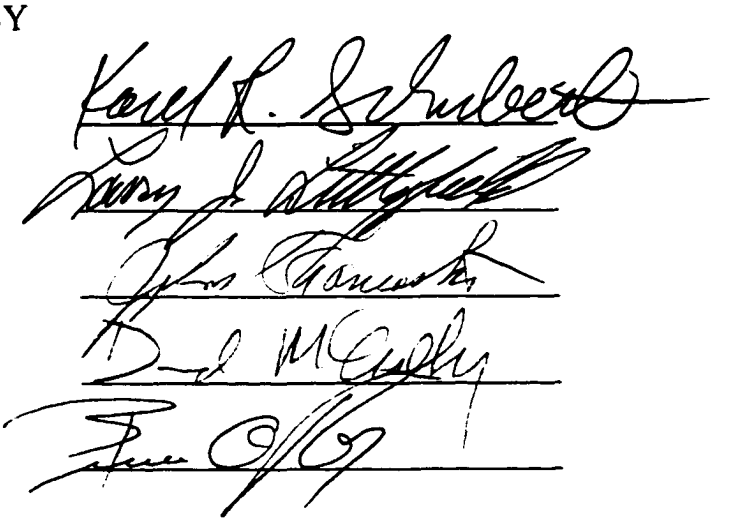
TAHZEEBA HOSSAIN

Norman, Oklahoma
1999

DISCOVERY, PURIFICATION AND CHARACTERIZATION OF ANTIFUNGAL PROTEINS AND OTHER DEFENSE-RELATED COMPOUNDS FROM TROPICAL PLANTS

A Dissertation APPROVED FOR THE
DEPARTMENT OF BOTANY AND MICROBIOLOGY

BY



DEDICATION

This dissertation is dedicated to six special people in my life who wholeheartedly encouraged me to follow my dream and to never give up. They are my daughter M. Tanzeena Hossain, my husband Mohammed M. Hossain, my late grandmother Mrs. Zamila Razzak, my mother Mrs. Taiyeba Rahman, my father Mr. A. H. M. Rahman and my major professor Dr. Karel R. Schubert.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Every year we loose billions of dollars in agriculture because of fungal pathogens. Chemical controls of these pathogens provide some protection but the continued use of chemical pesticides is under attack because of economic, environmental and health issues. The focus of our research is to find alternative ways to protect crop plants from fungal pathogens and pests. Our specific goal is to discover, purify and characterize antifungal proteins and to isolate genes encoding these proteins. Once isolated these genes can be engineered into crop plants to increase their resistance to fungal diseases. Our research has focused on plant species from the tropical rainforest because of the diversity of species present in these areas and because of the virtually untapped wealth of novel defenses used by these plants.

To date, we have screened 164 extracts from over 100 different plant species for antifungal activity against *Fusarium chlamydosporum*, a saprophytic/pathogenic fungus. Out of these 164 crude extracts, 111 had antifungal activity. Thirty-five aqueous crude extracts exhibited very strong to complete inhibition of growth of *F. chlamydosporum*. Forty of the 164 extracts retained activity after dialysis (3,500 molecular weight cut off) suggesting that proteins may be responsible for the activity. Five of the

dialyzed extracts showed strong to complete inhibition of fungal growth. Antifungal activities present in several of these plant extracts were purified further.

Novel antifungal activities were discovered in extracts from seeds of three different legumes, *Swartzia simplex*, *S. cubensis* and *Pentaclethra macroloba*. These activities included chitinases and other defense-related proteins and peptides. Chitinases were purified from all three sources using a combination of chitin-affinity chromatography followed by anion-exchange chromatography. Alternatively, chitinases were purified by a combination of affinity-chromatography followed by size-exclusion chromatography. Acidic chitinases isolated from all three species inhibited growth of *A. flavus* in a liquid bioassay.

The physical and biochemical properties of selected chitinases were determined. Purified chitinases were stable in 0.1 M HCl, 0.1 M acetic acid and 0.1 M NaOH. The molecular weight, pI, glycosylation, amino acid composition and partial amino acid sequence of several forms of the purified chitinases were determined. The partial amino acid sequence revealed a high degree (over 60%) of sequence homology to class three lysozyme/chitinase and endochitinase precursor from several plant species.

In addition to chitinases, other antifungal proteins/peptides were detected in all three species mentioned above. Agglutination and β-1,3-glucanase activities present in these extracts were associated with several fractions that inhibited the growth of *A. flavus*. Other novel activities were detected and purified. These activities await further characterization.

A protein-containing fraction purified from *Coccoloba* sp. exhibited a broad range of antifungal activity. This material strongly inhibited conidial germination and hyphal growth of *A. flavus*, *Fusarium chlamydosporum*, *F. moniliforme*, *Sclerotinia minor* and *Sclerotium rolfsii*. The antifungal activity was stable in 0.1 M NaOH, 0.1 M HCl, 0.1 M acetic acid, survived boiling for 5 min and was retained after dialysis using 3,500 to 14,000 molecular weight cut off dialysis membranes. Purified materials caused changes in fungal morphology including hyperbranching and extremely reduced hyphal elongation. These observations are consistent with the effects of many morphogenic plant defensins suggesting that the active component may be a defensin-like protein.

Chapter I : Introduction

Catastrophic losses in yield have occurred throughout the centuries because of fungal diseases. In 1845, the potato crop of Ireland was destroyed by late blight disease caused by *Phytophthora infestans*. This fungus caused a famine so devastating that half a million people died and one and a half million people migrated from Ireland (Agrios, 1997; Daly, 1996). The Great Bengal Famine of 1943 is another example of the devastation caused by fungal diseases. Famine occurred because the entire rice crop was destroyed by the fungal disease, brown spot of rice. Thousands died from starvation and starvation related diseases. Although not so directly devastating, other epidemics such as chestnut blight, Dutch elm disease and coffee rust have decimated whole species within a region (Agrios, 1997).

The indirect cost of such epidemics can be tremendous. Estimates in 1982 suggest that economic losses due to plant diseases in the United States approached $9.1 billion. The Southern corn leaf blight alone caused losses in 1970 in the range of $1 billion (Agrios, 1997). In the United States, the average disease losses in cotton are in the range of 15%. According to the Cotton Disease Council of America, the value of the cotton fiber lost in 1975-78 because of fungal wilts caused by *Fusarium* and *Verticillium* was

$74 million (Schnathorst, 1981). The United States is the world's largest exporter of soybean oil (Sinclair and Backman, 1989). Yield losses of soybean up to 59% have been reported from a single disease caused by *Fusarium* sp. (Datnoff, 1989). It is estimated that in the U.S., 10 million acres of soybean crops are lost each year from diseases caused by *Phytophthora* (Anon, 1986). Every year 600,000 hectors of peanuts with a value exceeding one billion dollars are harvested in the United States. Stem rot and *Sclerotinia* blight are two major fungal diseases of peanuts in major peanut producing states such as Oklahoma and Virginia. These diseases cause yield losses ranging from 10% to 80% in infected fields (Porter, 1990). When the cost due to yield losses is coupled with the cost of disease control, the impact of these diseases on human civilization is staggering.

Pests and pathogens of crop plants have typically been controlled through the application of chemical pesticides. Without the application of these pesticides, crop loss would increase by 10% and in the case of specific crops, losses would range from zero to nearly 100% (David, 1992). Worldwide, the cost of manufacture and application of chemical pesticides is in the billions of dollars. In the United States alone, the cost of pesticide treatment for peanuts and potatoes are $175 and $139 million, respectively. For peanuts, 90% of the acres are treated with fungicide (Table I-1). The

Table I-1: Value and Treatment Costs for Selected Crops in the United States.

CROP	ACREAGE	VALUE	% ACRES TREATED		TREATMENT COST
	Millions Acres	$ Billion	Insecticides	Fungicides	($ Million)
Corn	79	17	30	NA	700
Peanuts	1.8	2.1	60	90	175
Potatoes	1.35	2.5	90	70	139
Cotton	13	4.1	67	6	576
Soybean	60	12	2	NA	NA

Source: Agricultural Statistics 1994, U. S. Department of Agriculture.

total consumer cost of pest control products in 1995 reached $32 billion with fungicides accounting for $6.5 billion. The market value of pesticides has increased from $300 million in 1950 to the present value of $32 billion in 1995. Projections indicate that the value of pest control will increase another 50% by the year 2010 (Figure I-1).

The use of chemical pesticides has increased drastically due to changes in agricultural practices and consumer demand for esthetically pleasing produce. Western Europe and the United States are the major users of synthetic pesticides, accounting for 31% and 26% of the total usage, respectively (Chatterjee, 1994). Since 1945, pesticide use in the U.S. has increased dramatically. In 1995, 1.2 billion pounds of synthetic pesticides were applied to crops in the U.S. compared to 540 million pounds in 1964 (Environmental Protection Agency, 1996). Although synthetic pesticide use has reached an all time high, their continued widespread use is under scrutiny. Conventional pesticide application is not very efficient with only 1% of the active component actually reaching the targeted pest. The remaining 99% of the pesticide applied are wasted. The wasted pesticides

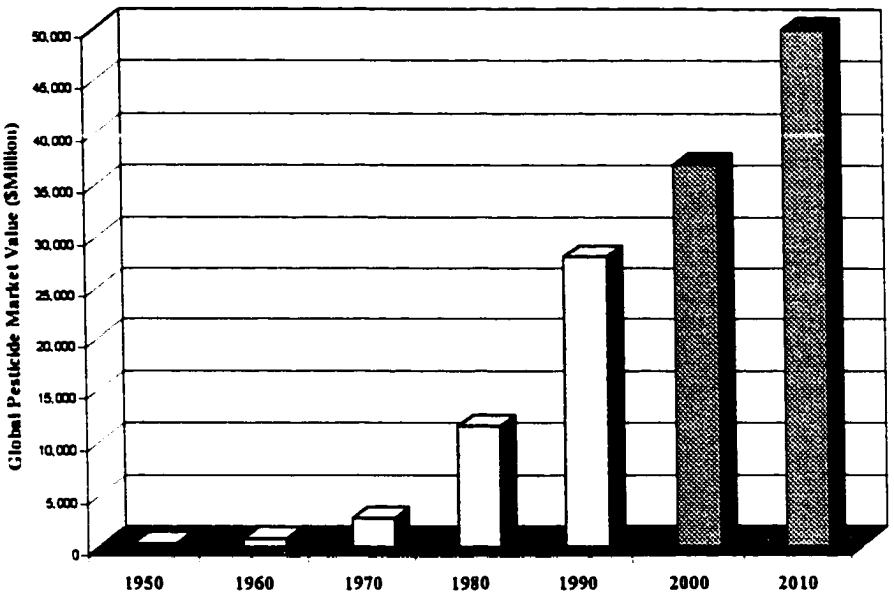


Figure I-1: Global Value of Pest Control Market. Values for 2000 and 2010 are projected values. Values are taken from Global Insecticide Directory (First Edition), by W. Hopkins, Ag Chem Information Services, Indianapolis, Indiana (1996).

and their residues contaminate soil, water supplies, produce and wildlife (Martinez, 1990).

Half the population of the United States relies on groundwater for their drinking water supply (Nader, 1988). An Environmental Protection Agency (EPA) survey found 23 pesticides in the groundwater in 24 states. According to an United States Department of Agricultural (USDA) Marketing Service survey of pesticide use on produce, the frequently used fungicide, Chlorothalonil, was applied on green beans an average of 3 to 4 times in 1992. Out of 36% of the samples of Chlorothalonil-treated green beans tested, 7.1% of the samples contained residue of the fungicide. Captan is the most heavily used fungicide on apples and 52% of the apple crop receives an average of 7.3 treatments per season with Captan. The same USDA survey found Captan in 7.3% of the samples of Captan-treated apples. The Agricultural Marketing Service survey screened 568 potato samples and found 530 cases of pesticide contamination from 16 different chemicals (Kuchler, 1996).

Environmental pollution by the fungicide pentachloronitrobenzene (PCNB) was investigated in Japan. High concentrations of PCNB were detected in the river water near a cabbage farming area. Principal PCNB biodegradation products such as pentachloroaniline (PCA) and

pentachlorothioanisole (PCTA) were also found in the river water and in river sediment. The study indicated a higher rate of conversion of PCNB to PCA in the river sediments than in the river water. According to this study the principal PCNB biodegradation products remain in the environment for a long time and accumulate in river sediment and soil (Fushiwaki, 1990). The effect of the fungicide orthocide on certain desirable microorganisms such as Azotobacter, nitrogen-fixing bacteria etc. was studied. Lower growth of all microorganisms was observed in fungicide-treated soil (Makawi et al., 1979).

Many routinely used synthetic pesticides are carcinogenic in nature and can produce cancerous tumors in humans. The EPA has data on 74 of the 289 currently (1990) registered pesticides. Out of these 74 pesticides, 53 were classified as oncogenic and in total these 53 compounds accounted for 90% of all fungicides used (Martinez, 1990). Approximately one third of all the fruits and vegetables produced in the United States are treated with a class of fungicides which break down into a carcinogenic compound upon heating (Nader, 1988).

Tetrachloroisophthalonitrile (TCPN) is a fungicide used in many parts of the world. This compound is also used as a wood preservative in Northern Europe. This toxic fungicide causes allergic contact dermatitis

(Johnson, 1983). Fluazium, the active ingredient of the fungicide Shirlan, was responsible for the outbreak of contact dermatitis in a tulip bulb processing company in the western part of the Netherlands (Van Ginkel and Sabapathy, 1995). In the United States, contact dermatitis due to the active fungicidal chemical dyrene was reported in 1997 (Mathias, 1997). These are just a few of the examples of carcinogenic, environmentally unfriendly and toxic fungicides that are used in agriculture today. As the use of these fungicides increases, so do the public concerns of the harmful effects of these chemicals.

Another major concern is that monocroping, a practice prevalent in the industrialized nations, along with the continued application of the same chemical control year after year has led to the selection of populations of pests and pathogens that have developed resistance to these chemical pesticides, making it necessary to apply even greater amounts of these pesticides. According to the Congressional Record, 447 species of insects and mites are resistant to members of all principal classes of insecticides. According to the same report, there are 35 species of weeds and 100 species of plant pathogens which are resistant to chemical pesticides (Proxmire, 1986). It has been estimated that the increased resistance to pesticides costs U. S. farmers at least $118 million annually (Knight and Norton, 1989).

Chemical controls are extremely costly and substantial losses occur in spite of pesticide application. A survey conducted by Oerke *et al.* indicated that crop loss due to diseases, weeds and insects have increased in both developed and underdeveloped countries in spite of better and widely used chemical controls (Oerke et al., 1994). The combined costs of pest control and crop losses are passed on to the consumer. Only a small fraction of the pesticide reaches the target. Most of the compounds end up in the soil or ground water. Pests and pathogens develop resistance to these compounds, making these chemicals even less effective. Many synthetic pesticides are hazardous to man and the environment, therefore, a number of pesticides have been banned from use in developed countries. Without any chemical protection or other safe pest control alternatives, agricultural productivity will be devastated by pests and pathogens.

Alternative strategies need to be developed to protect plants from pests and pathogens, to decrease environmental pollution, and to minimize the development of pesticide resistant pests. Genetic engineering and biotechnology provide such an opportunity. Through genetic engineering and biotechnology, foreign genes may be introduced into plants to protect these crops from pests and pathogens. Genetically-engineered resistance to insects, fungal pathogens, and viruses has been achieved by expression of Bt

toxin (Wood and Granados, 1991) or trypsin inhibitor (Chen, 1998), chitinase and glucanase (Broglie et al., 1991; Jongedijk et al., 1995; Lin et al., 1995; Zhu et al., 1994), and viral coat protein genes, respectively. These techniques have already been proven to be very successful. In 1993 the USDA has issued 158 permits to test genetically engineered crops (Cox, 1993).

Genetic engineering offers many advantages. These include:

1. Single gene for resistance can be incorporated into the plant without the transfer of deleterious or undesirable genes and subsequent genetic drag.
2. Genes can be highly specific for selected pests so beneficial and non-parasitic organisms are not affected.
3. Gene expression can be targeted to specific tissues, e. g. the roots, leaves or seeds and/or developmental stages, e. g. during sensitive stages of development such as germination, early seedling growth or seed formation.
4. Gene expression can be localized to specific cellular and subcellular sites, e. g. extracellular.
5. Gene expression can be constitutive or inducible, for instance triggered upon infection or attack. The later insures that the protective agent is

present only when needed e. g. during initial infection, thereby, limiting the damage done and reducing the cost to the host plant for defense.

6. The cost of application is eliminated. This is very important for crops such as wheat, produced on large acreage with relatively low inputs and yields.
7. Genetic engineering allows for gene transfer from any organism, avoiding the sexual barriers that limit conventional breeding.

Plants can be protected from fungal pathogen by the introduction and expression of antifungal genes. The major factor limiting the exploitation of genetic engineering and biotechnology is the discovery of pesticidal genes. There are several different approaches to the discovery of antifungal genes. One needs to either identify and sequence the gene product, i. e. an antifungal protein, and use this information to isolate the gene or use approaches to directly clone the gene, e. g. using sequence homology, mutational or combinatorial studies. We have chosen to screen tropical plants for antifungal proteins. Antifungal proteins exist in nature but to discover these proteins, it may be necessary to screen a large number of plants. Once an antifungal protein is discovered, the next step is to isolate and characterize that protein, so that the gene expressing this protein can be

cloned and characterized. Once the gene is cloned and characterized, it can be introduced into plants via techniques of plant transformation.

The goal of this research project was to discover, isolate and characterize antifungal proteins from tropical plants. Once purified and characterized the information gained can be used as the basis for cloning the gene encoding the antifungal protein. Ultimately, this gene can be inserted into crop plants via various mechanisms and transgenic plants expressing this gene can be tested for their ability to resist fungal attack. In the end, the transgene should confer protection from attack by fungal pathogens.

Chapter II : Review of Literature

INTRODUCTION

Plants have developed an arsenal of defense mechanisms to protect themselves from pests and pathogens. Various compounds are synthesized by the plant defense system either to prevent pathogen attack or to destroy the pathogen after infection. These compounds include proteins as well as lower molecular weight natural products. Many of these compounds are produced constitutively while others are induced by pathogen attack. The production of these antimicrobial compounds may also be induced by stress or different chemicals such as ethylene, jasmonic acid and salicylic acid.

Defense-related proteins produced by plants can be categorized into three major groups based on their role in plant defense. The first group includes proteins that are involved in strengthening the structural defenses of a plant. These proteins strengthen, alter or repair the cell wall of plants creating a physical barrier to ward off pathogens. Examples of proteins in this group include hydroxyproline-rich glycoproteins, glycine-rich proteins and various enzymes that are involved in the formation or modification of cell wall polymers such as lignin, suberin, callose and cell wall bound phenolics.

The second group of proteins is directly involved in the destruction of the invading pathogens. These antimicrobial proteins include amylase inhibitors, proteinase inhibitors; toxic proteins such as lectins and thionins; hydrolases such as chitinases and β-1,3-glucanases; anti-microbial peptides such as defensins and other cysteine-rich proteins. Enzymes involved in the biosynthesis of antibiotic compounds such as phytoalexins may be placed in this group. The third class of proteins is known as pathogenesis-related proteins or PR proteins. The appearance of these proteins is correlated with fungal attack and the host plant's defense responses such as the hypersensitive reaction of plants to pathogen attack.

Not all PR proteins exhibit activity against fungal pathogens. PR proteins that have been shown to possess antifungal properties include chitinases, β-1,3-glucanases, osmotins and several other chitin-binding proteins, e. g. PR-1 and PR-4. Although the identity of some PR proteins has been established, the biological functions of many PR proteins are still unknown. There can be an overlap between proteins placed in group 2 and group 3. For example, chitinases and glucanases are placed in both groups. The major distinction seems to be whether the production of a protein is induced by the pathogen.

Our research goal has been the identification of plant antifungal proteins that are directly active in the host plant's defense against fungal pathogens. Consequently, this literature review will focus only on the latter two groups. The properties of proteins involved in strengthening the structural defenses of plants will not be discussed. Likewise, biosynthetic enzymes involved in the synthesis of antibiotic compounds will not be included. The structure and functions of selected PR proteins, defensins, lectins, thionins, ribosome-inactivating proteins and nonspecific-lipid-transfer proteins will be emphasized.

PATHOGENESIS-RELATED PROTEINS

Pathogenesis-related proteins were first detected in tobacco leaves infected with tobacco mosaic virus (Van Loon, 1970). Since 1970, a number of PR-proteins have been isolated from tobacco and characterized. Although the largest number of PR-proteins isolated up to now are from tobacco, PR-proteins have also been purified from other plant species, both dicots and monocots (Stintzi et al., 1993).

The synthesis of PR-proteins is induced by both biotic and abiotic factors. Viruses, viroids, fungi and bacteria are among the biotic factors that induce PR-protein production in plants (Benito et al., 1998; Bol et al., 1990;

Buchter et al., 1997; Dubery and Slater, 1997; Hu et al., 1997; Lawrence et al., 1996; Mohr et al., 1998; Stintzi et al., 1993; Van Loon, 1985). Members of these groups are also induced by chemicals such as salicylic acid, aspirin, jasmonic acid, heavy metals, etc. (Chen et al., 1994; Kan et al., 1995; Kim et al., 1996; Linthorst, 1991; Schneider Muller et al., 1994; Schraudner et al., 1992; Siefert and Grossmann, 1997; Siefert et al., 1996). Osmotic stress, wounding, stress hormones like ethylene and other phytohormones may also induce expression of many PR-proteins (Chen and Bleecker, 1995; Chen et al., 1994; Keefe et al., 1990; Mauch et al., 1992; Mauch and Staehelin, 1989; Sagee et al., 1995). Developmentally regulated expression of PR-proteins has been observed in roots, senescent leaves and flowers of tobacco.

Typically, PR proteins are 1) stable at extremes of pH; 2) relatively resistant to digestion by proteolytic enzymes; and 3) normally exist as monomers of low molecular weight (8 to 50 kDa). Because of these typical physiochemical properties, pathogenesis-related proteins survive in harsh environments such as vacuolar compartments, the cell wall and intercellular spaces (Stintzi et al., 1993).

Because PR-proteins have been isolated and characterized from many plant sources, it has been useful to develop a classification scheme for PR-proteins. At the 3rd International Workshop on PR-proteins, a common

classification and nomenclature was agreed upon to facilitate the study of these proteins. PR-proteins were grouped into five families, i. e. PR-1, PR-2, PR-3, PR-4 and PR-5. Representatives from these five families seem to be ubiquitous in the most commonly studied plants. In this section, the properties and role of these five groups of proteins in plant defense will be reviewed.

PR-1 Proteins

Tobacco PR-1 was the first pathogenesis-related protein isolated. Since that time, both acidic and basic forms of PR-1 have been isolated from tobacco and other plant species (Joosten et al., 1990; Naderi and Berger, 1997; Niderman et al., 1995; Pierpoint, 1986; Szybiak Strozycka et al., 1995). Acidic PR-1a, 1b and 1c from tobacco are serologically related to each other and to PR-1 from other plant species (Antoniw et al., 1985; Nassuth and Sanger, 1986; White et al., 1987). PR-1 cDNA clones isolated and sequenced from tobacco show a high degree of sequence similarity. For example, sequence homology between PR-1a, 1b and 1c was approximately 90%. PR-1 proteins of tobacco are encoded by a small gene family consisting of at least eight genes [for a review see (Bol et al., 1990; Linthorst, 1991; Stintzi et al., 1993)].

Although PR-1 proteins are induced by viral infection, transgenic plants expressing high levels of PR-1 cDNA did not exhibit resistance to TMV or alfalfa mosaic virus (Cutt et al., 1989; Linthorst et al., 1989). However, the antifungal properties of PR-1 proteins isolated from TMV-infected tomato and tobacco were clearly demonstrated by Niderman et al. (1995) in both *in vitro* and *in vivo* leaf disc assays (Table II-1). These PR-1 proteins were found to display differential and dose dependent antifungal activity against *Phytophthora infestans*. Among the PR-1 proteins tested, basic PR-1's from tomato (P14c) and tobacco (PR1-g) were found to be more active against *P. infestans* than the acidic PR-1 proteins from the same plants. A minimum concentration of over 100 μg/ml of acidic PR-1 proteins was required to inhibit germination of *P. infestans* zoospores by 90% while only 20 μg of basic PR-1 proteins was required to give the same level of inhibition in the *in vitro* assay.

Additional evidence for the antifungal properties of PR-1 proteins was provided by studies involving the expression of PR-1 cDNA in transgenic plants. Constitutive high level expression of PR-1a in transgenic tobacco resulted in tolerance of the transgenic plants to two oomycete pathogens, *P. parasitica* and *Peronospora tabaciana* (Alexander et al., 1993). Although the exact mechanism of tolerance is not known, it was proposed that PR-1a

either 1) exerts a direct fungicidal effect that decreases disease development in transgenic plants or 2) reduces pathogen establishment and aids in pathogen recognition until other defense components are activated.

Table II-1: Properties of PR-1 Proteins Isolated from Tobacco and Tomato

Protein	Plant Source	Molecular Weight (kDa)	Isoelectric Point	Biological Activity*
PR1-a	Tobacco	15.5	< 7	Antifungal
PR1-b	Tobacco	15.5	4.5	Antifungal
PR1-c	Tobacco	15	4.7	Antifungal
PR1-g	Tobacco	17	> 7	Antifungal
PR14a	Tomato	14	10.7	Antifungal
PR14b	Tomato	14	10.9	Antifungal
PR14c	Tomato	14	> 10.7	Antifungal

*Antifungal activity against *Phytophthora infestans* has been observed in PR-1 proteins from tobacco and tomato (Joosten et al., 1990; Niderman et al., 1995; Stintzi et al., 1993).

PR-2 Proteins

β-1,3-glucans are polysaccharides consisting of β-1,3-linked glucose monomers. PR-2 proteins are β-1,3-glucanases that hydrolyze laminarin, an essentially unbranched β-glucan and/or other β-glucans. Most β-1,3-glucanases are endoglucanases, which produce oligomers of 2 to 6 glucose units. The occurrence of endo-β-1,3-glucanases is widely distributed among angiosperms. Glucanases from various plant species have been purified, cloned and sequenced (Meins et al., 1992).

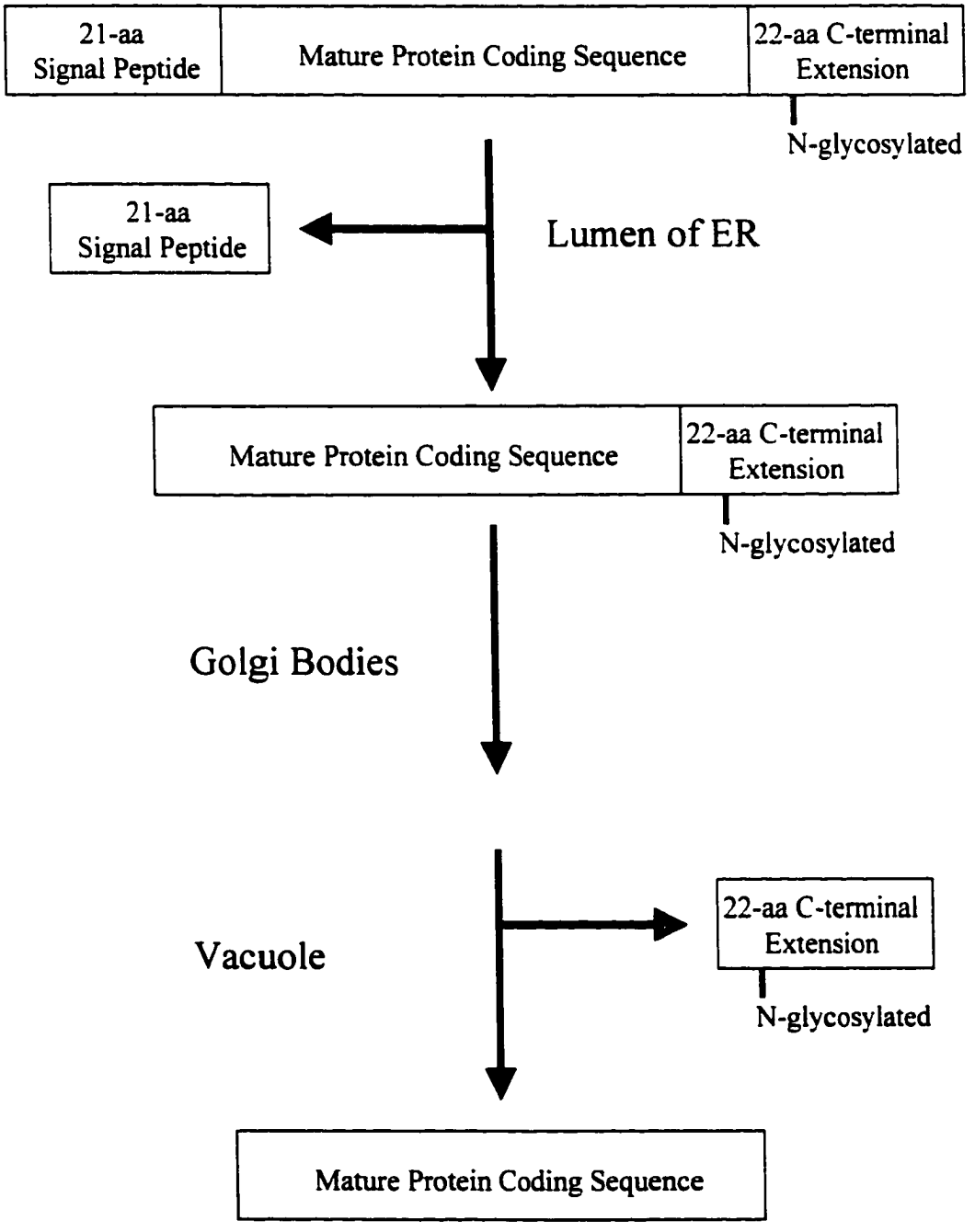
Plant β-1,3-glucanases isolated and characterized so far have been grouped into three structural classes (Payne et al., 1990; Ward et al., 1991). Class I β-1,3-glucanases are basic vacuolar glucanases while class II and III glucanases are extracellular acidic glucanases. Class II glucanase from different plant species are closely related to tobacco PR-2, PR-N and PR-O proteins and class III glucanases are closely related to β-1,3-glucanase PR-Q from tobacco. Among the three classes of β-1,3-glucanases, tobacco class I β-1,3-glucanases have been studied in the greatest detail.

Tobacco class I β-1,3-glucanases are located in the vacuole of mesophyll and epidermal cells (Keefe et al., 1990; Van den Bulcke et al.,

1989). The post-translational processing of tobacco class I β-1,3-glucanase is depicted in Figure II-1. Structurally, Class I isoforms of β-1,3-glucanase are synthesized as a preproenzyme with a 21 amino acid N-terminal signal peptide and a N-glycosylated 22 amino acid C-terminal extension peptide (Shinshi et al., 1988; Sticher et al., 1992). The signal peptide is responsible for targeting the protein to the lumen of the ER. The proprotein is then transported to the vacuole via the Golgi apparatus, where the proglucanase undergoes post-translational modification and the C-terminal extension is removed to form the mature class I β-1,3-glucanase (Figure II-1). The process is analogous to the processing of tobacco class I chitinase (Neuhaus et al., 1991) and barley lectins (Bednarek and Raikhel, 1991) where the C-terminal extension is a sorting signal for vacuolar targeting (Worrall et al., 1992). Southern blot analysis of tobacco genomic DNA indicated that the tobacco class I glucanases represent a small gene family of less than eight genes (Beffa and Meins, 1996).

Analysis of genomic southern blots probed with cDNA clones of class II glucanases indicated that there were eight genes encoding class II tobacco glucanases (Linthorst et al., 1990). Although class II and III glucanases are

Figure II-1: Structure and Processing of β–1,3 Glucanase Preproprotein



both acidic and are located extracellularly, comparison of the deduced amino acid sequence of these two classes of glucanases from tobacco revealed only 54-59% homology. Class III glucanases of tobacco also differ from the basic class I glucanases and comparison of the protein sequence deduced from the cDNA for both classes exhibited 55% sequence homology. Comparison of the C-terminal end of basic class I and acidic class III glucanases provides insight into a possible mechanism for removal of the C-terminus of class I glucanase that involves recognition and cleavage at a conserved phenylalanine-glycine site. The sequence for the carboxy-terminal processing site for class I glucanase was phenylalanine-glycine. This dipeptide is the C-terminus of mature class III glucanase (Payne et al., 1990).

Plant glucanases are involved in physiological and developmental processes such as microsporogenesis (Worrall et al., 1992), pollen germination (Rogen and Stanley, 1969), fertilization (Lotan et al., 1989), and seed germination (Vogeli-Lange et al., 1994). Plant glucanases also play a role in plant defense. The substrate for glucanase (β-1,3-glucan) is a major component of most fungal cell walls (Wessels and Sietsma, 1981). Induction of plant glucanases in response to fungal invasion has been observed in maize (Cordero et al., 1994), barley (Ignatius et al., 1994), carrot

(Cam et al., 1994), potato (Godoy et al., 1996; Kombrink et al., 1988), tomato (Solorzano et al., 1996), pea (Mauch et al., 1988) and several other plant species (Roulin and Buchala, 1995). In addition, β-1,3-glucanases are also induced by elicitors, chemicals, phytohormones and abiotic stress as observed with other PR proteins (Boller et al., 1983; Hwang et al., 1997; Mauch and Staehelin, 1989; Siefert and Grossmann, 1997; Siefert et al., 1996; Kombrink et al., 1988; Roby et al., 1990; Schneider and Ullrich, 1994; Sock et al., 1990).

Specific isoforms of β-1,3-glucanases are induced by fungal pathogens and other kinds of biological and non-biological stresses (Nasser et al., 1990). Differential regulation, induction and expression of β-1,3-glucanases were observed in response to fungal pathogens, tobacco mosaic virus and abiotic stress (Brederode et al., 1991; Cordero et al., 1994; Mamelink et al., 1990). Hormonal, developmental and pathogen induced regulation of tobacco glucanase promoter was demonstrated by Vogeli-Lange and co-workers (Vogeli-Lange et al., 1994). In this case, the class I β-1,3-glucanase promoter was fused with a GUS reporter gene and expressed in transgenic tobacco. According to Vogeli-Lange et al. (1994), glucanases were expressed in roots of young seedlings and preferentially expressed in the lower leaves and roots in mature plants. Induction of

glucanase expression by ethylene treatment or inoculation with tobacco mosaic virus was observed in leaves. Tissue specific expression and differential expression of β-1,3-glucanases in response to fungal invasion was demonstrated by Cordero et al. (1994). According to these investigators, β-1,3-glucanase was induced in the vegetative tissues of maize seedlings after inoculation of germinating maize seeds with the corn pathogen *F. moniliforme*. At the same time, expression of another isoform of β-1,3-glucanase was observed in embryo and radicle tissue. However, the level of this glucanase did not increase upon fungal infection.

In response to TMV infection, class II and class III glucanases in tobacco were coordinately regulated in infected and uninfected tissue. The kinetics and pattern of induction for both classes of β-1,3-glucanase were qualitatively similar to the accumulation to mRNA encoding other PR proteins in infected and uninfected tissues. In uninoculated tissue, the induction of class I glucanases was weak compared to class II glucanases (Ward et al., 1991). Inoculation of potato leaves with *Phytophthora infestans* caused induction of class I β-1,3-glucanase along with chitinases (Beerhues and Kombrink, 1994).

Class I and class II β-1,3-glucanase was purified from tobacco leaves infected with TMV (Sela-Buurlage et al., 1993). When tested for antifungal activity, purified class I β-1,3-glucanase was found to be the most active glucanase isoform against *Fusarium solani*, causing lysis of hyphal tips. Class II β-1,3-glucanase from the same plant did not show any inhibitory activity against the same pathogen. Class I glucanases also exhibited synergistic activity with class I and class II chitinases.

Antisense transformation using the tobacco gene encoding class I β-1,3-glucanase was used to confirm the function of plant β-1,3-glucanases in plant defense (Beffa and Meins, 1996). Although the expression of the class I β-1,3-glucanase gene was blocked, the transformed plant compensated by producing functionally equivalent enzymes when treated with tobacco mosaic virus and ethylene. In addition, the transformed plants were fertile and developed normally in the greenhouse, suggesting that the expression of class I β-1,3-glucanase blocked by the antisense construct was not important for housekeeping functions of healthy tobacco cells.

Research on plant β-1,3-glucanases clearly indicates that the antifungal activity of plant β-glucanases varies with different isoforms. Differences in the activity may reflect differences in substrate specificity.

For example, tobacco PR-2 glucanases differed in substrate specificity towards laminarin and β-1,3-glucan (Table II-2). When tested for substrate specificity using *P. megasperma* cell wall, purified glucanase from soybean was very active whereas tobacco glucanase did not show any significant activity toward this substrate (Ham et al., 1991). This is logical since *P. megasperma* is a pathogen of soybean but does not cause disease in tobacco. A 19-kDa barley β-1,3-glucanase differed greatly from typical laminarinase (Grenier et al., 1993). This particular β-1,3-glucanase did not hydrolyze laminarin, alkali soluble β-1,3-glucan, and β-1,6-glucan but did lyse *Candida* yeast and fission yeast cell wall (Grenier et al., 1993). It is not clear why some β-1,3-glucanases are lytic to cell walls and some are not. According to Grenier et al. (1993), one possible explanation is that the lytic β-1,3-glucanases have high affinity for insoluble wall glucan.

Although some isoforms of glucanases do not exhibit direct antifungal activity, it is possible that they act as elicitors or release elicitors and induce the host plant's defenses. For example, the constitutive β-1,3-glucanase isolated from soybean releases soluble and highly active carbohydrate elicitors from fungal cell wall. Four different soybean elicitors released by

Table II-2: Properties of PR-2 Proteins (β-1,3-Glucanases).

Name	Class	Molecular Weight (kDa)	Isoelectric Point	Relative Specific Activity (%)
PR-2	II	31	4.4	0.4
PR-N	II	33	4.7	1.8
PR-O	II	35	4.8	85
PR-Q'	III	35	5.3	3.8
	I	33	7	100

Recreated from Stintzi et al., 1993.

treatment of *P. megasperma* mycelia with β-1,3-endo-glucanase were purified and characterized (Okinaka et al., 1995). These elicitors were composed of a β-1,6-linked glucose backbone with frequent β-1,3-linked side branches consisting of one or two glucose moieties.

It has been proposed that vacuolar class I enzymes function late in the infection process after cell breakage and release of cellular contents into the extracellular matrix has occurred. Induction of these glucanases is correlated with pathogen invasion and they are usually strongly induced at the site of infection. Class I enzymes may also act as a constitutive defense system in plants since these enzymes accumulate in the epidermal cells of uninfected plants (Felix and Meins Jr, 1986; Payne et al., 1990). Upon wounding, these enzymes would be released providing protection from invading fungi entering through the wound site. Other non-vacuolar isoforms may act directly on the fungus, depending on substrate specificity, or may activate the plant's defense system by releasing elicitors from the fungal or plant cell wall and therefore, still play an important role in plant defense.

PR-3 Proteins

Chitin is one of the most abundant polysaccharides found in nature. Chitin, a linear polymer of β-1,4-linked N-acetyl glucosamine, is a common component of insect exoskeleton, shells of crustaceans and fungal cell walls. Chitin-containing organisms produce chitinase, an enzyme that hydrolyzes the C_1-C_4 bond of two consecutive N-acetylglucosamine units of chitin. Chitinases are also produced in bacteria and higher plants, organisms that are devoid of chitin. Legrand and co-workers demonstrated that four different PR-3 proteins exhibited chitinase activity (Legrand et al., 1987). Since then, many plant chitinases have been purified and characterized. Interest in plant chitinases has increased dramatically over the last 12 years, as the significance of chitinases in plant defense and plant microbe interactions has become evident. Ultimately, this has led to the publications of a large numbers of papers. In the present review, the discussion will focus mainly, on the role of chitinases, in plant defense against pathogenic fungi.

Most of the chitinases isolated to date are endochitinases (Graham and Sticklen, 1994). Endochitinase hydrolyzes chitin internally by cleaving the β–1,4-linkages and producing short chitooligosaccharides of two to six N-acetylglucosamine units. On the other hand, exochitinase activity has only been observed in a few plant chitinases (Kuzniak and Urbanek, 1993;

Martin, 1991; Nehra et al., 1997; Nielsen et al., 1993; Roby and Esquerre-Tugaye, 1987; Wurms et al., 1997). Acidic chitinase purified from sugar beet leaves infected with *Cercospora beticola* exhibits both exo and endochitinase activity. This chitinase was capable of hydrolyzing chitooligosaccharides into N-acetylglucosamine monomers. Along with chitin-hydrolyzing ability, many plant chitinases possess lysozyme activity, i.e. these chitinases can hydrolyze the β-1,4-linkage between N-acetylmuramic acid and N-acetylglucosamine of bacterial peptidoglycans.

Some chitinases are produced constitutively while others are inducible. Chitinases can be induced in higher plants by 1) pathogen infection; 2) elicitors, e. g. chitooligosaccharides from the fungal cell wall, plant cell wall or other sources; 3) chemicals such as ethylene, salicylic acid, mercuric chloride; and 4) physical stress such as wounding (for a review see (Graham and Sticklen, 1994). Chitinases produced constitutively have been purified from *Hevea* and *Papaya* latex (Azarkan et al., 1997; Martin, 1991), banana pulp (Clendennen et al., 1998) and *Castanea crenata* cotyledons (Collada et al., 1993). In healthy plants, chitinases are also produced in a developmental and a tissue specific manner. High levels of chitinase are found in roots and flowers of these plants. Chitinases are also present in

seeds, fruits and leaves, although the level of chitinases in healthy leaf tissue is usually low relative to roots and flowers (Trudel et al., 1989).

Acidic and basic chitinases and the genes encoding these proteins have been isolated from monocots and dicots (Table II-3). Characterization of these proteins and genes has resulted in the accumulation of substantial amounts of information on the protein, cDNA and genomic sequences (Table II-4). Chitinases purified and characterized so far are divided into three classes based on sequence homology, biochemical properties and localization in the plant cell (Shinshi et al., 1990).

Most tobacco class I chitinases have basic isoelectric points above 8. Class I chitinases possess a N-terminal cysteine-rich, chitin-binding domain of 40 amino acids. This N-terminal domain exhibits structural similarity with chitin-binding lectins such as hevein and wheat germ agglutinin (Figure II-2). Certain positions in this cysteine-rich domain are highly conserved, i. e. eight cysteine residue are present at the same position in the chitin-binding region of almost all class I chitinases. Next to the cysteine-rich domain is a glycine and proline-rich domain (glycine and arginine in rice) which connects the cysteine-rich domain to the chitin-binding domain. The length of the glycine-proline-rich domain varies in different class I chitinases (Graham and Sticklen, 1994).

Table II-3: Properties of Chitinases Isolated from Monocotyledenous and Dicotyledenous Plants.

Plant Source	Isoform	Molecular Weight (kDa)	Isoelectric Point	Class
Tobacco	4	27.5-34	ND	I, III
Tomato	7	26-34	6.1 to 8.5	I, III
Potato	9	26.5-38.7	6.1 to 7	I, III
Pea	5	25-39	8.5-9.3	I
Sugarbeet	2	32	ND	III
Soybean	1	29.0	3.0	ND
Wheat	5	33.5-34	5.8-9.2	ND
Barley		26-35	8.7-9.7	I, II
Maize	4	25-33	ND	I, II
Garlic	1	32	ND	I
Yam	3	33.5	3.8-4.0	ND
Melon	2	29, 34	8.4, 10.0	I
Cucumber	2	27, 28	ND	III

Different classes and isoforms of chitinases can exist in a single species. Chitinases can be either acidic or basic and typically exist as monomers of 25 to 35 kDa. ND, not determined. Reference: Tobacco (Legrand et al., 1987); Tomato (Breijo et al., 1990; Joosten and De Wit, 1989; Pegg and Young, 1982); Potato (Kombrink et al., 1988; Pierpoint et al., 1990); Pea (Mauch et al., 1988; Vad et al., 1991); Sugar beet (Esaka et al., 1990); Soybean (Nielsen et al., 1993); Wheat (Molano et al., 1979; Ride and Barber, 1990); Barley (Jacobsen et al., 1990; Kragh et al., 1990; Kragh et al., 1991; Leah et al., 1987; Leah et al., 1991; Swegle et al., 1992); Maize (Nasser et al., 1988); Garlic (Van Damme et al., 1993); Yam (Tsukamoto et al., 1984); Melon (Roby and Esquerre-Tugaye, 1987); Cucumber (Metraux et al., 1989).

The catalytic domain is 61% homologous at the amino acid sequence level between tobacco, potato and bean class I chitinase (Graham and Sticklen, 1994). The catalytic domain contains a 20-residue hypervariable region along with several conserved cysteine residues that may be involved in the formation of a loop (Meins et al., 1992).

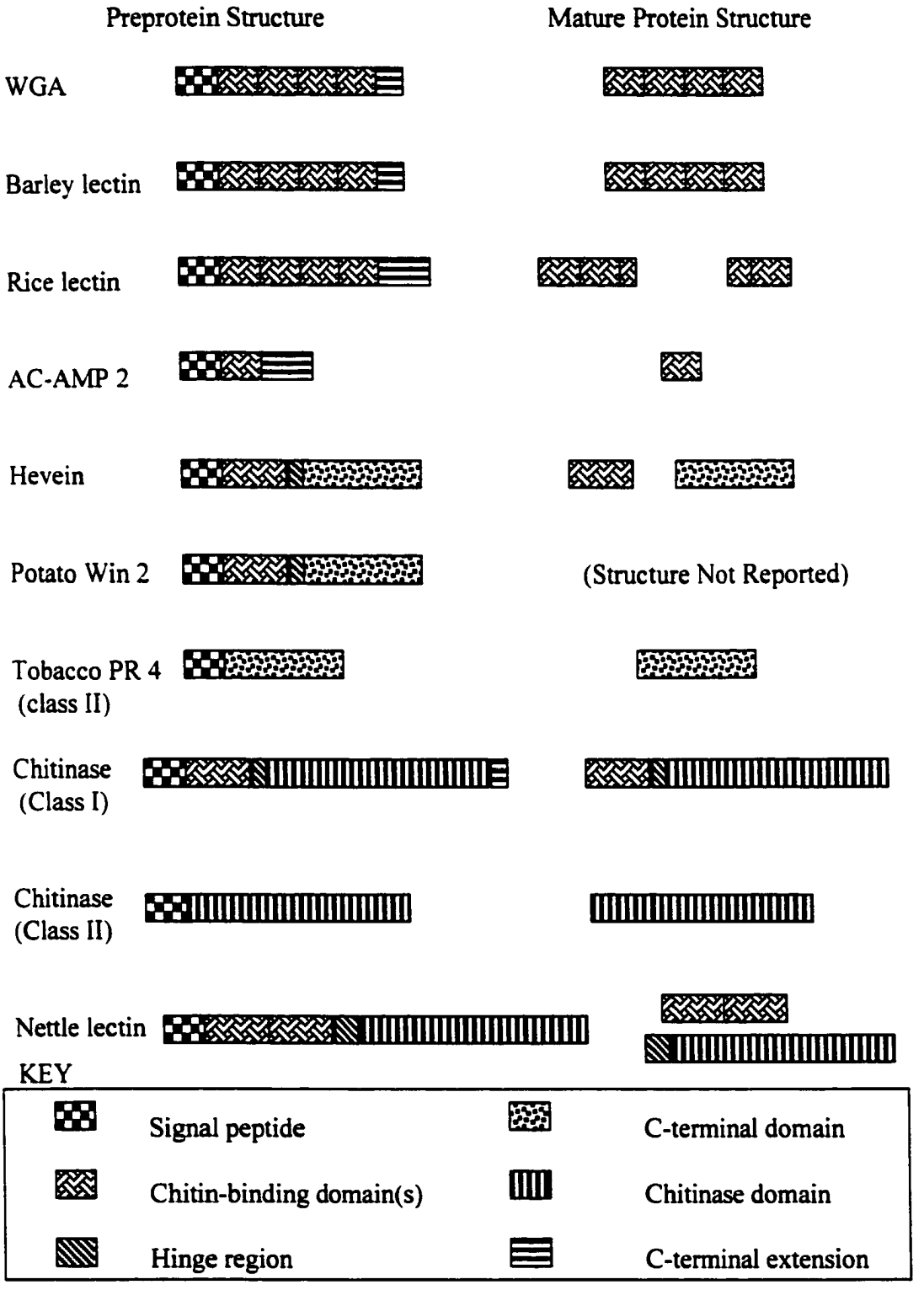
Class I chitinases are synthesized as a preproprotein with a signal peptide that may have some role in directing the protein to the endoplasmic reticulum. This peptide is removed from the mature protein by post-translational modification. The length of this signal peptide is different in different class I chitinases (Broglie et al., 1991; Shinshi et al., 1990; Zhu and Lamb, 1991). Class I chitinases are usually located in the vacuole. A short C-terminal amino acid sequence is necessary for vacuolar targeting of class I chitinases (Chrispeels and Raikhel, 1992; Neuhaus et al., 1991). In tobacco class I chitinase, a seven amino acid C-terminal sequence (GLLVDTM) was necessary and sufficient for vacuolar targeting (Neuhaus et al., 1991).

Class II chitinases do not have the N-terminal cysteine-rich domain characteristic of class I chitinases (Figure II-2). Class II chitinases consist of a catalytic domain and a signal peptide. The signal peptide is hydrophobic

Table II-4: Summary of References on the Protein, cDNA and Genomic Sequences of Plant Chitinases.

Plant	Class	Sequence	Reference
Tomato	I, II	Protein, cDNA	(Danhash et al., 1993)
Potato	I	Protein, cDNA	(Gaynor, 1988)
		Genomic	(Gaynor and Unkenholz, 1989)
			(Laflamme and Roxby, 1989)
	II	Partial protein	(Pierpoint et al., 1990)
Rice	I	Protein	(Zhu and Lamb, 1991)
		Genomic	(Huang et al., 1991)
		cDNA	(Nishizawa and Hibi, 1991)
Tobacco	I	Protein, cDNA	(Fukuda et al., 1991)
		Genomic	(Neale et al., 1990)
			(Shinshi et al., 1990)
			(Van Buuren et al., 1992)
	II	Protein, cDNA	(Linthorst, 1991)
			(Payne et al., 1990)
	III	Protein, cDNA	(Lawton et al., 1992)
Sugar beet	III	Protein, cDNA	(Nielsen et al., 1993)
Cucumber	III	Protein, cDNA	(Metraux et al., 1989)
		Genomic	(Lawton et al., 1994)
Garlic	I	Protein, cDNA	(Van Damme et al., 1993)
Bean	I	Protein, cDNA,	(Broglie et al., 1989; Broglie et
		Genomic	al., 1986)
Barley	I	Protein, cDNA	(Jacobsen et al., 1990; Kragh et
	II	Protein, cDNA	al., 1991; Leah et al., 1991)

Figure II-2: Primary Structure of Selected Chitin-Binding Proteins



in nature and is generally 23 amino acids long. The catalytic domain of class II chitinases shows strong sequence homology to the catalytic domain of class I chitinases. These chitinases are generally acidic and are located in the apoplastic compartment. The signal peptide of class II chitinases differs substantially from the signal peptides of class I chitinases.

Class III chitinases can be either acidic or basic and are compartmentalized in the extracellular space (Flach et al., 1992). Class III chitinases do not show any sequence homology or serological relationship with class I or II chitinases. Some members of class III chitinases have limited similarity to *Bacillus circalan* chitinase I (Watanabe et al., 1992). Several class III chitinases exhibit lysozyme activity (Table II-5).

The antifungal activity of several of plant chitinases has been demonstrated using an *in vitro* assay (Table II-6). Differential antifungal activity was observed among the three classes of chitinases and within the members of the same class. For example, two class I chitinases (Chit A and Chit B) from maize seeds showed differential *in vitro* antifungal activities even though they were very similar at the amino acid level (i.e. 87% sequence homology). Although Chit A and B both exhibited antifungal activity against *Trichoderma reesi, Alternaria solani* and *Fusarium solani* Chit A exhibited better activity than Chit B.

Table II-5: Hydrolases with Chitinase and Lysozyme Activities.

Plant Source	**Purified as**	**MW (kDa)**	**Reference**
Bean	Chitinase	31.5	(Boller et al., 1983)
Cucumber (seeds)	Chitinase	27.0	(Majeau et al., 1990)
Figure (Ficus sp.)	Lysozyme	29.0	(Glazer et al., 1969)
Hevea (latex)	Chitinase	27.5, 26.0	(Martin, 1991)
Papaya (fruit)	Lysozyme	28	(Howard and Glazer, 1969)
Parthenocissus quinquifolia (leaves)	Chitinase-lysozyme	30	(Bernasconi et al., 1987)
Pea (pods)	Chitinase	33.1, 36.2	(Mauch et al., 1988)
Rubus hispidus	Lysozyme	31.3	(Bernasconi et al., 1986)
Turnip (root)	Lysozyme	25	(Bernier et al., 1971)

Modified from Graham and Sticklen, 1993

Table II-6: Antifungal Activity of Plant Chitinases.

Plant Source	**Fungus**	**Reference**
Bean	*Trichoderma viridae*	(Mauch et al., 1988; Schlumbum et al., 1986)
Wheat	*Trichoderma hamatum* *Phycomyces blakesleeanus*	(Broekaert et al., 1988)
Tobacco	*Trichoderma hamatum* *Phycomyces blakesleeanus* *Fusarium solani*	(Broekaert et al., 1988) (Sela-Buurlage et al., 1993)
Thorn apple	*T. hamatum* *Phycomyces blakesleeanus*	(Broekaert et al., 1988)
Arabidopsis	*Trichoderma reesei*	(Verburg and Huynh, 1991)
Pea	*Trichoderma viridae*	(Mauch et al., 1988)
Maize	*Fusarium oxysporum* *Alternaria solani* *Trichoderma reesei*	(Huynh et al., 1992)
Barley	*Trichoderma reesei* *F. sporotrichoides*	(Leah et al., 1991)

Antifungal activity of chitinases isolated from different plant species was assessed using pathogenic and saprophytic fungi. Antifungal activity was estimated based on inhibition of spore germination or hyphal growth.

The binding constant (Kd) of Chit A and Chit B was determined using the soluble chitinase substrate, N,N′,N″,N‴-tetraacytyl chitotetrose. The binding constant for Chit B (1.9 mM) was 10-fold higher than that for Chit A (0.13 mM). This observation suggests that Chit A may have higher affinity for chitin. Therefore, Chit A is more likely to bind effectively to the chitin in the fungal cell wall. Fungal growth was inhibited with only 0.5 μg of Chit A whereas 5 μg of Chit B was required to inhibit fungal growth. Sela-Buurlage and associates demonstrated that not all plant chitinases from tobacco exhibit antifungal activity (Sela-Buurlage et al., 1993). Tobacco class I chitinase inhibited growth of *F. solani* by hydrolyzing hyphal tips of the fungus. On the other hand, class II chitinase from tobacco did not exhibit any antifungal activity against the same fungus. Based on these observations, it appears that class I plant chitinases, play a direct role in plant defense.

As stated earlier, the major difference between class I chitinase and the other two classes of chitinase is that class I chitinase has a N-terminal cysteine-rich, chitin-binding domain. The importance of the chitin-binding domain in fungal susceptibility was studied (Iseli et al., 1993). This research group constructed a transformation vector with genes of tobacco chitinase A

with or without a chitin-binding domain. These genes were constitutively expressed in transgenic tobacco plants. The expressed chitinases were purified to homogeneity from the transgenic plant and tested for *in vitro* antifungal activity. Chitinases with and without a chitin-binding domain were capable of inhibiting growth of *T. viride*. When tested for chitin-binding ability, chitinase A without the chitin-binding domain was unable to bind to a chitin-affinity matrix but both forms of chitinase (with and without chitin-binding domain) exhibited similar specific hydrolytic activity towards chitin. These results indicate that the chitin-binding domain was not necessary for catalytic activity.

However, the presence of the chitin-binding domain does modify the biochemical properties of the protein. For example, the chitinase form with the chitin-binding domain had higher activation energy and a lower apparent Km for chitin. Although both forms of chitinase inhibited fungal growth, the antifungal activity of chitinase with the chitin-binding domain was three times more effective on a protein basis. Based on these results, these authors concluded that the catalytic domain of class 1 chitinase is sufficient for inhibition of fungal growth but the addition of the chitin-binding domain improves the antifungal activity. Apparently, chitinases have a high affinity binding site and a low affinity catalytic site. Iseli and associates proposed

that the chitinase with the chitin-binding domain had higher activity because the chitin-binding domain aids in the initial attachment of the enzyme to the substrate. This increases the local concentration of chitin around the catalytic site and at the same time increases the local concentration of chitinase on the cell wall of the fungi.

Even though class I and class II chitinases have the same catalytic domain antifungal activity has been mostly associated with class I chitinases. Even so class II chitinase with lower specific activity may still play an indirect role in plant defense. These chitinases may act as a signaling molecule to trigger plant defense directly or by releasing elicitors from the fungal cell wall rather than destroying the fungus. Exposure of plant cells to chitooligosaccharides has been shown to induce chitinase activity (Fukamizo et al., 1996; Inui et al., 1997; Roby and Esquerre-Tugaye, 1987). Extracellular class II chitinases may initially come in contact, with the fungal cell wall forming the first line of defense. Without destroying the fungus, these chitinases may release fragments of the fungal cell wall capable of triggering other plant defenses.

Antifungal activity of specific chitinases is correlated with the structure of fungal cell walls. In the case of maize, barley and several other plants, the antifungal effect of chitinases depends on the fungi tested. For

example, *Arabidopsis* chitinase exhibited antifungal activity against *T. reesi* but not against *A. solani* (Verburg and Huynh, 1991). Likewise, chitinases from tobacco, thornapple and wheat inhibited growth of *T. hamatam* but did not affect *Botrytis cineria* (Broekaert et al., 1988). In all of the examples listed above, the fungi have chitin in their cell wall. Indeed, the two major carbohydrate components of the cell wall of a majority of fungi, especially fungi with septate mycelia, are chitin and glucan (Bartnicki-Garcia, 1968).

However, the amount of chitin can vary in each fungus. According to Verbug and Huynh, the amount of chitin in the fungal cell wall determines the susceptibility of the fungus to the action of chitinase (Verburg and Huynh, 1991). Quantitative data on the composition of various fungal cell walls is limited. Rokem and associates observed that the carbohydrate composition of fungal cell wall was quite variable (Rokem et al., 1986). For example, the ratio of N-acetylglucosamine to glucose was 2.5:1 for *Marchella crassipes* cell wall whereas the ratio was 1.5:5 in the case of *Candida utilis* cell wall.

Protein, cDNA, and genomic sequences of several plant chitinase have been described (Table II-4). The cDNA clone encoding acidic class III chitinase isolated from a sugar beet cDNA library from plants infected with the sugar beet pathogen *Cercospora beticola* has been constitutively

expressed in tobacco (Nielsen et al., 1993). Transgenic plants did not show any increased resistance to *C. nicotianae*. Even so, these plants accumulated higher levels of chitinase mRNA. Roby and associates demonstrated activation of a bean chitinase gene (CHI 5B) promoter in transgenic tobacco upon attack by fungal pathogens *Botrytis cineria, Rhizoctonia solani* and *Sclerotium rolfsii* (Roby et al., 1990).

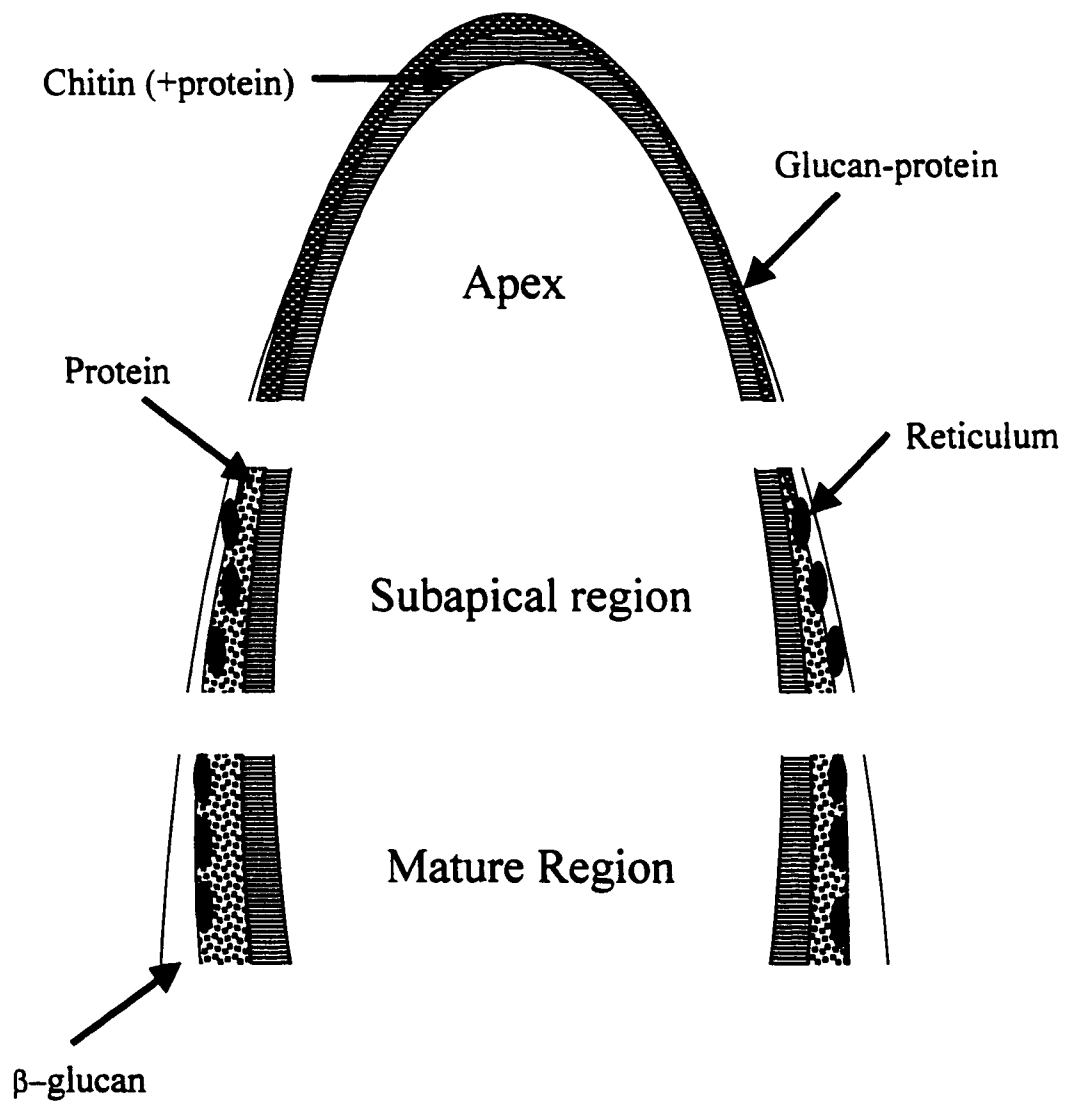
Broglie and co-workers produced transgenic tobacco plants by constitutively expressing CHI 5B, a bean chitinase gene, under the control of the cauliflower mosaic virus 35S promoter (Broglie et al., 1991). The gene was correctly expressed in tobacco plants producing a high level of chitinase activity. According to Broglie et al. (1991), the highest chitinase activity was found in the roots of the transgenic tobacco seedlings. These seedlings showed increased ability to survive in soil infested with the soil-borne plant pathogen *R. solani*. Seedling mortality was 37% in transgenic plants compared to 56% in the untransformed seedlings.

The bean chitinase gene was also introduced into canola. Transgenic canola plants exhibited 50% mortality compared to 80% mortality of wild type canola in *R. solani* infested soil. Transgenic rice plants constitutively expressing rice class I chitinase exhibited enhanced protection from *R. solani*, the causative agent of sheath blight of rice (Lin et al., 1995).

Compared to untransformed control plants, transgenic plants infected with the fungus had fewer lesions and the lesions were smaller. Three weeks after infection, disease symptoms had spread to the upper half of the control plants. In contrast, transgenic plants remained free of infection on the upper half of the plant, especially the flag leaves that contribute most to grain filling.

Although reports about the role of plant chitinases in plant defense has increased tremendously over the past 10-15 years, very little is known about the mechanism of fungal growth inhibition by chitinase. Chitinase-dependent hydrolysis of fungal cell wall polysaccharides has been reported by several research groups (Arlorio et al., 1992; Boller, 1985; Kuzniak and Urbanek, 1993). A morphological study of fungal growth inhibition by plant chitinase and glucanases was carried out by Arlorio and associates (Arlorio et al., 1992). In this study, observations made by combined use of light microscopy, fluorescence microscopy, scanning electron microscopy and transmission electron microscopy revealed that chitin was present in the apex of *T. viride* (Figure II-3). After treatment of fungal cell wall with chitinase, the hyphal wall became swollen and detached chitin fibrils were already visible. Gradually the walls became thinner and eventually disappeared. Without the structural strength of the cell wall the hyphal tip

FigureII-3: Diagrammatic Representation of a Typical Fungal Cell Wall.

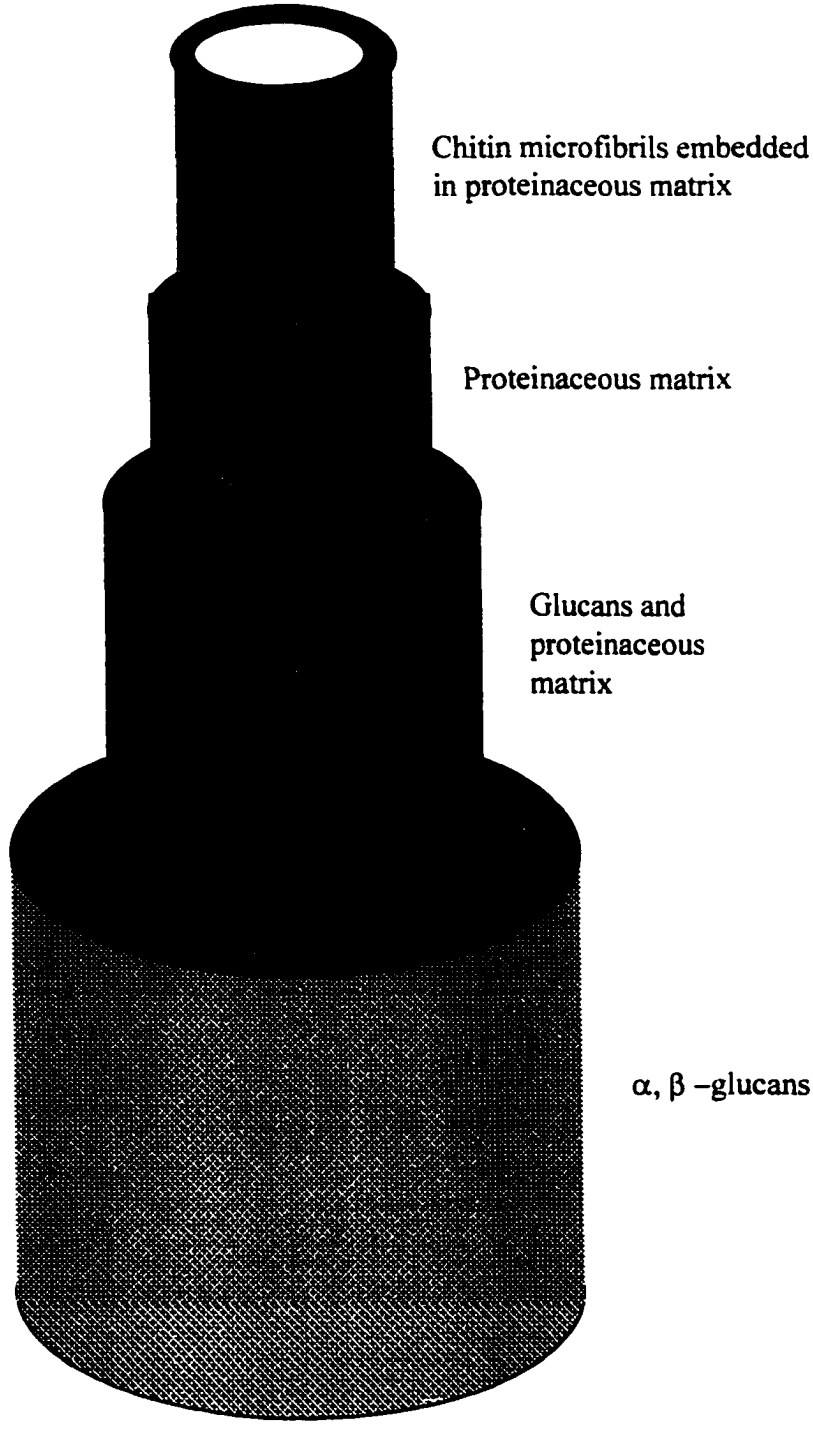


expanded like a balloon and finally the plasma membrane ruptured. According to Arlorio et al. (1992), chitinase did not interfere with the cell wall synthesis machinery but simply weakened the cell wall at the hyphal tip and altered the cell's ability to withstand turgor pressure. Thus, chitinases inhibit by disturbing the balance between cell wall synthesis and degradation. The formation of balloon-like swellings in fungal hyphae followed by rupture of hyphal tips proved to be the cause for the susceptibility of *T. viridi* and *F. solani* f. sp. *pisi* to bean chitinase (Mauch et al., 1988).

Synergistic antifungal activity of β-1,3-glucanases and chitinases

As stated earlier, chitin and glucan are the two major components of the fungal cell wall (Figure II-3 and Figure II-4). These carbohydrates are susceptible to hydrolysis by chitinases and glucanases, respectively. Synergistic effect of cucumber chitinase and β-1,3-glucanase against *Colletotrichum lagenarium* in an *in vitro* assay has been reported by Ji and Kuc (Ji and Kuc, 1996). The synergistic activity of pea chitinases and glucanases was demonstrated by Mauch et al. (1988).

Figure II-4: Diagrammatic Representation of the Structure of the Primary Cell Wall of a Typical Fungus



Pea chitinase exhibited antifungal activity against *T. viridi* and pea glucanase showed antifungal activity against *F. solani* f. sp. *pisi* when tested alone. In combination, these two enzymes inhibited growth of eight different pathogenic and/or saprophytic fungi in an *in vitro* assay at concentrations from 10 to 30 μg/ml.

In a similar study, Seela-Buurlage et al. (1993) reported on the synergistic activity of tobacco chitinases and glucanases. Class II isoforms of both enzymes did not exhibit any antifungal activity against *F. solani* when tested alone. When class II chitinases were combined with class I β-1,3-glucanases, limited growth inhibitory activity was observed. A combination of class I chitinase and class I β-1,3-glucanase was the most effective at inhibiting fungal growth.

Transgenic plants expressing a combination of chitinase and glucanase genes have been used to demonstrate the synergistic effects of chitinase and glucanase. Zhu and associates (Zhu et al., 1994) demonstrated enhanced protection in transgenic tobacco to *Cercospora nicotianeae* (causal agent of frog eye disease) by constitutive co-expression of basic chitinase and acidic glucanase genes from rice. Enhanced disease resistance of transgenic tomato by simultaneous expression of tobacco class I chitinase and class I β-1,3-glucanase was achieved by Jangedijk et al. (Jongedijk et al., 1995). In this

study transgenic tomato plants infected with *F. oxysporum* showed reduced (36%) disease severity compared to 58% in untransformed plants.

Using cytochemical techniques in combination with fluorescence and electron microscopy, Arlorio et al. (1992) observed the combined effects of chitinase and glucanase on fungal cell wall. This study revealed that chitin was present at the tip of the fungal hyphae and along the lateral part of the inner cell wall whereas, glucans were present in the outer cell wall (refer to Figure II-3 and II-4). The results of these morphological studies on the effects of chitinase and glucanase on the fungal cell wall help to explain the synergistic effects of these two hydrolases. To begin, the degradation of the outer wall glucan by glucanase would make the chitin at the apex more susceptible to the action of chitinase. In addition, the effectiveness of chitinase in hydrolyzing the inner wall a chitin-glucan complex may be enhanced by the simultaneous activity of glucanase.

PR-4 Proteins

Properties of PR-4 proteins from various plant sources are presented in Table II-7. Two isoforms of PR-4 proteins were isolated from tobacco leaves infected with TMV (Friedrich et al., 1991). These proteins known as PR-4a and PR-4b are acidic in nature, have molecular masses of 13.4 kDa and are located extracellularly. The deduced amino acid sequence of these two PR-4 proteins showed 75% homology to the carboxy-terminal domain of the wound-induced potato proteins (Win-1 and Win-2) and hevein, a protein from rubber tree latex. The amino acid sequence deduced from the cDNA sequence revealed that PR-4 proteins are synthesized as preproteins with signal peptides. The signal peptide of tobacco PR-4 protein is comprised of about 25 amino acid residues. The same is true for Win-1, Win-2 and hevein. However, unlike Win 1, Win-2, and hevein, these acidic PR-4 proteins do not contain a chitin-binding domain (Figure II-2).

Similar but basic counterparts of PR-4a and PR-4b have been purified from tomato leaves infected with *Cladosporium fulvum* (Joosten et al., 1990). A 13.7 kDa basic PR-4 protein (CBP-N) was purified from barley grain (Hejgaard et al., 1992). Two very similar basic PR-4 proteins (CBP-4

Table II-7: Biochemical Properties of PR-4 Proteins from Selected Plant Sources.

Protein	**Plant source**	**Molecular Weight (kDa)**	**Isoelectric point**	**Antifungal activity**
PR-4a	Tobacco	13.4	6.2	ND
PR-4b	Tobacco	13.4	6.2	ND
PR-4a	Tomato	15	7	ND
PR-4b	Tomato	15	7	ND
PR-4c	Tomato	15	7	ND
CBP-N	Barley	13.6	9.3	*T. herzianum*
CBP-4	Barley	13.6	9.3	*T. herzianum*
CBP-5	Barley	13.7	9.3	*T. herzianum*
CBP-20	Tobacco	20		*T. viride* *F. solani*

Antifungal activity was measured using an *in vitro* assay. ND; not determined.

and CBP-5) were isolated from barley leaves infected with *Erysiphe graminis*. All three PR-4 proteins from barley exhibited high amino acid sequence homology to tomato and tobacco PR-4 proteins and to the C-terminal domain of prohevein and the putative Win proteins. The three barley proteins exhibited antifungal activity against *Trichoderma herzianum* at a concentration of 10 μg/ml (Hejgaard et al., 1992). The ability of these proteins to retard fungal growth was equivalent to the combined effects of chitinase and PR-5 isolated from barley. All three PR-4 proteins from barley acted synergistically to inhibit fungal growth when combined with barley chitinase and the barley PR-5 known as protein R. The PR-4 proteins from barley leaves are inducible while the PR-4 protein from barley grain (CBP-N) is produced constitutively and deposited along with chitinase in the outer aleurone layer of the grain. In nature, it is possible that the combined action of all of these proteins, are required to efficiently retard fungal attack. Although the three barley PR-4 proteins, like their acidic counterparts, do not possess a chitin-binding domain, they were isolated by chitin-affinity chromatography and exhibited antifungal activity against chitin-containing fungi. These proteins do not exhibit chitinase or lysozyme activity.

A 20 kDa PR-4 protein (CBP 20) was purified by Ponstein and co-workers (Ponstein et al., 1994) from TMV infected tobacco leaves. Like

class I chitinases, this protein was located intracellularly. CBP 20 contained an N-terminal chitin-binding domain as observed in class I chitinases, Win-1 and Win-2 of potato and prohevein from rubber tree. In all these proteins, the chitin-binding domain was preceded by a signal peptide and connected to the C-terminal domain by a hinge region (Figure II-2). CBP 20 is processed to form the mature protein by removal of the signal peptide and a C-terminal propeptide. Typically, class I proteins are localized in the vacuole of the plant cell and class II proteins are usually present extracellularlly. On this basis, all the extracellular PR-4 proteins lacking a chitin-binding domain were classified as class II PR-4 proteins. The PR-4 proteins with a chitin-binding domain and a C-terminal propeptide were classified as class I PR-4 proteins (Linthorst et al., 1991). The C-terminal domain of class I PR-4 protein CBP 20 exhibited strong similarity (75-78%) to all the class II PR-4 proteins (Linthorst, 1991; Ponstein et al., 1994). Southern blot analysis of a tobacco genomic library using PR-4 cDNA as a probe indicated that the PR-4 proteins are encoded by a relatively small gene family comprised of 2 to 4 members (Friedrich et al., 1991; Linthorst, 1991; Ponstein et al., 1994).

According to Ponstein et al. (1994), CBP 20 exhibited antifungal activity toward *T. viridi* and *F. solani* by causing lysis of the germ tube and/ or inhibition of hyphal growth. *T. viride* appeared to be more sensitive to

this protein than *F. solani*. This protein also acted synergistically with tobacco class I chitinase to inhibit growth of *F. solani* and with tobacco class I β-1,3-glucanase to inhibit both *F. solani* and *Alternaria radicana*.

PR-5 Proteins

A fifth group of pathogenesis-related proteins, PR-5 proteins, have been identified. PR-5 proteins are highly homologous to the sweet-tasting protein thaumatin extracted from the African-rainforest shrub *Thoumatoccus donielli*. For this reason, PR-5 proteins are also known as thaumatin-like proteins (Cornelissen et al., 1986; Edens et al., 1982). The occurrence of thaumatin-like proteins is widespread in nature. These proteins are found in both dicotyledenous and monocotyledenous plants (Bryngelsson and Green, 1989; Choi et al., 1997; Hejgaard et al., 1991; Huynh et al., 1992; Van Loon, 1985; Vigers et al., 1992; Vu and Huynh, 1994; Woloshuk et al., 1991).

Slightly acidic to neutral tobacco PR-5 protein R (pI 6.9) and neutral tobacco PR-5 protein S (pI 7.5) were among the 10 PR proteins initially detected using a native basic PAGE system (Van Loon, 1985; Van Loon, 1970). Tobacco protein R and protein S share greater than 90% sequence identity and both of these proteins are very similar (60% sequence homology) to thaumatin (Cornelissen et al., 1986; Payne et al., 1988; Singh

et al., 1989). Among the five different forms of thaumatin isolated from *Thoumatoccus donielli*, two forms (thaumatin I and II) are found to be more abundant (Cornelissen et al., 1986). Thaumatin I and II are 207 amino acids in length, and differ from each other only at five positions. Thaumatins are synthesized as preprothaumatins with an amino-terminal signal peptide of 22 amino acids and an acidic carboxy-terminal extension of 6 amino acids. The mature protein is obtained after removal of the signal peptide and the C-terminal extension (Cornelissen et al., 1986).

Two serologically related basic counterparts of tobacco R and S were purified and identified as osmotins (Nelson et al., 1992; Stintzi et al., 1993). Osmotins were first detected in osmotically-stressed cell suspension cultures of tobacco (King et al., 1986). Two basic osmotins were found in the cells subjected to salt (NaCl) stress. Osmotin I was soluble in aqueous solution and osmotin II was solublized only after detergent treatment. The osmotins are very similar in structure to thaumatins. Both thaumatins and osmotins are hydrophobic in nature, have similar molecular weights, contain high proportions of proline and are stabilized by several disulfide bonds.

PR-5 proteins have been purified from various plant species (Table II-8). The cDNA clones corresponding to acidic and basic PR-5s of tobacco

have been isolated (Hu and Reddy, 1997; Liu et al., 1996; Nelson et al., 1992; Payne et al., 1988; Rodrigo et al., 1993; Singh et al., 1989; Zhu et al., 1995). The basic PR-5 proteins were located in the vacuole and the neutral and slightly acidic forms were extracellular and localized in the apoplast (Nelson et al., 1992; Singh et al., 1989). Like other pathogenesis-related proteins, some PR-5 proteins are induced by fungal infection and salicylic acid (Hu and Reddy, 1997; Woloshuk et al., 1991). Thaumatin and several other PR-5 proteins from the Graminae are produced constitutively (Vigers et al., 1991; Walden et al., 1990).

Several PR-5 proteins exhibited direct and differential antifungal activity in *in vitro* assays. Woloshuk et al. (1991) demonstrated that osmotin II had antifungal activity against pathogenic oomycetes. Osmotin II isolated from TMV inoculated tobacco leaves caused lysis of *P. infestans* sporangia and inhibition of fungal growth. Osmotin (AP 24) purified from *P. infestans* infected tomato leaves exhibited similar antifungal activity against *P. infestans* in the *in vitro* assay.

Two thaumatin-like proteins isolated from barley grain exhibited homology to the bi-functional α-amylase/protease inhibitor from maize grain (Hejgaard et al., 1991). These two proteins named protein R and protein S were as potent as ribosome-inactivating protein K from barley.

Table II-8: Antifungal Properties and Activities of Various PR-5 Proteins.

Name	**Molecular weight (kDa)**	**Source**	**Pathogen**	**Reference**
Osmotin	24	Tobacco	*P. infestans* *C. albicans* *T. reesi* *N. crassa*	Woloshuk et al., 1991 Vigers et al., 1992
Zeamatin	22	Maize	*C. albicans* *F. oxysporum* *T. reesi* *N. crassa* *A. solani*	Roberts et al.1990 Quang et al., Huynh et al., 1992
Trimatin		Wheat	*C. albicans* *N. crassa* *T. reesi*	Vigers et al., 1992
Protein R	23	Barley	*C. albicans* *T. viride* *F. oxysporum*	Hejgaard et al., 1991
Protein S	23	Barley	*C. albicans* *T. viride* *F. oxysporum*	Hejgaard et al., 1991
Thaumatin		*T. donielli*	*C. albicans*	Vigers et al., 1992
PR-5	25	Flax	*A. solani* *C. albicans*	Borgmeyer et al.,1992
PR-S	24	Tobacco	*C. beticola*	Woloshuk et al, 1991 Vigers et al., 1992
PR-5	27	*D. taxana*	*P. infestans*	Vu and Huynh, 1994

Both proteins inhibited growth of *F. oxysporum*, *T. viride* and *Candida albicans*. Although protein R and S were homologous to α-amylase/proteases inhibitor, they did not inhibit α-amylase or serine proteases. The barley grain proteins also acted synergistically with nikkomycin Z to retard fungal growth. Nikkomycin Z is a nucleoside peptide antibiotic active as competitive inhibitor of chitin synthase. Because of its structural resemblance to chitin monomer it inhibits the biosynthesis of fungal cell wall. Synergistic antifungal activity of barley proteins R and S in combination with barley chitinase C or barley ribosome-inactivating protein R was also observed.

Zeamatin, a twenty-two kDa PR-5 isolated from corn, exhibited antifungal activity against *Neurospora crassa*, *T. reesei* and *C. albicans* alone or in combination with nikkomycin Z (Huynh et al., 1992; Roberts and Selitrennikoff, 1990; Vigers et al., 1991; Walden et al., 1990). Synergistic antifungal activity with nikkomycin was also noted with PR-5 proteins isolated from other members of Poaceae family. The PR-5 proteins trimatin, avematin and sormatin were isolated from wheat, oat, and sorghum, respectively. These proteins inhibited growth of *C. albicans* when combined with nikkomycin Z (Vigers et al., 1991). These three proteins have

considerable amino acid sequence homology with each other and with zeamatin (Malehorn et al., 1994; Vigers et al., 1991). A cDNA clone encoding a zeamatin-like protein (Zlp) was isolated from a *Zea mays* cDNA library (Malehorn et al., 1994). The Zlp gene encoded a protein nearly identical with zeamatin and α-amylase/trypsin inhibitor. Despite the near identity of purified Zlp to α-amylase/trypsin inhibitor, Zlp proteins do not inhibit bovine trypsin (Malehorn et al., 1994). Zlp cDNA was expressed in insects, tobacco and *Arabidopsis* and the Zlp protein purified from all three sources exhibited antifungal activity against *C. albicans* and *T. reesei* (Malehorn et al., 1994).

Not much is known about the mechanism of action of PR-5 proteins. Based on the very hydrophobic nature of these proteins, Woloshuk et al. (1991) proposed that these proteins reacts with the plasma membrane of oomycetes causing cellular disruption and lysis of hyphae. In support of this hypothesis, the less hydrophobic isoform of PR-5 from tomato, AP 24, was less active against *P. infestans* when compared to the more hydrophobic isoform of AP 24. Zeamatin, trimatin, avematin and sormatin also exhibited membrane permeabilizing activity (Vigers et al., 1991; Walden et al., 1990).

Zeamatin did not hydrolyze chitin, glucan, mannan or proteins that are common components of fungal cell walls (Walden et al., 1990). When

fungal cells were treated with zeamatin, hyphal rupture was rapid and occurred in less than 15 seconds at 23°C (Walden et al., 1990). Hyphal rupture was also observed after treatment of different fungi with trimatin, avematin, and sormatin or other zeamatin-like proteins from seeds of several plant species (Vigers et al., 1991). Based on these findings, it appears that the mode of action of zeamatins does not involve hydrolysis of fungal cell wall. Instead, zeamatin may act directly by inserting itself into fungal membranes to form transmembrane pores. This conclusion has led to the suggestion that this family of membrane-permeabilizing antifungal proteins be referred to as prematins (Vigers et al., 1991; Walden et al., 1990). Fungal plasma membrane has also been identified as the target site for antifungal PR-5 protein purified from the flower bud of Chinese cabbage (Choi et al., 1997).

This kind of membrane-permeabilizing activity has been observed with proteins and polypeptides isolated from various sources. For example, polypeptides from bee venom (melittens), toad skin (magainins), haemolymph of insects (cecropins) and proteins such as the bacteriocins, colicin and halocin, appear to bind to the cell wall through cationic interactions and insert a hydrophobic domain through the lipid bilayer of the membrane (Bhakdi and Tranum-Jensen, 1987; Mackler and Kareil, 1977;

Parker et al., 1989; Steiner et al., 1981; Torreblanca et al., 1989; Zasloff, 1987). An analysis of the crystal structures of zeamatins suggests that all thaumatin-like proteins have an electrostatically polarized surface which may be critical for the antifungal activity of these proteins (Batalia et al., 1996).

A transmembrane protein kinase, PR5K, isolated from *Arabidopsis* was reported to be structurally related to tobacco acidic extracellular PR-5 protein (Wang et al., 1996). PR5K kinase, like other receptor proteins presumably binds to polypeptide ligands. This observation has lead to the suggestion that PR-5 proteins may interact with specific proteins, possibly polypeptides on the fungal cell surface. The binding of PR-5 proteins to cell surface proteins represent an entirely different mode of action from the hydrolytic enzymes such as chitinases and glucanases and other chitin-binding proteins which interact with cell wall carbohydrates.

OTHER DEFENSE–RELATED PROTEINS

Defensins

Plant defensins are a class of cysteine-rich, antimicrobial peptides. Individual members of this group differ in the number of disulfide bonds, mass and/or tertiary structure (Boman, 1995). These plant defensins

structurally resemble the structure of mammalian and insect defensins. Unlike linear antimicrobial peptides such as mammalian and insect cecropins, the plant defensins have extensive disulfide linkages forming a complex, disulfide-bond stabilized, three-dimensional structure. Therefore, the term plant defensins is commonly used to describe these cysteine-rich peptides from plants.

The first examples of plant defensins were isolated from wheat and barley and were referred to as γ-thionins because of their size similarity (5 kDa) with α and β-thionins. Later work has shown that the γ-thionins differ structurally from the α and the β-thionins. Since then, many plant defensins and defensin gene have been isolated and sequenced from taxonomically divergent plant species. Comparison of the amino acid sequence of different plant defensins reveals some common features (Broekaert et al., 1995). Most plant defensins are 45-54 amino acids in length, carry a net positive charge and show clear, although limited, sequence conservation. Conserved residues include eight cysteine residues, two glycine residues at position 13 and 34, an aromatic residue at position 11 and glutamate at position 29 (numbering relative to radish defensin, Rs-AFP1).

The amino acid sequences of seed defensins from *Amaranthus*, *Capsicum* and *Briza* are highly homologous to the chitin-binding domain of

plant chitin-binding proteins (Broekaert et al., 1996). The mature defensins from *Amaranthus* are essentially just a chitin-binding domain (Figure II-2). The deduced amino acid sequence from cDNA clones of different seed defensins revealed that the peptides are expressed as preproteins with N-terminal signal peptides (Bolle et al., 1993; Broekaert et al., 1996; Terras et al., 1995). Differences between defensin isoforms from the same plant species are minor. *Amaranthus* defensins AcAMP-1 and AcAMP-2 differ in a single amino acid residue and the later has one extra amino acid residue at the carboxy-terminal end (Broekaert et al., 1992). In the case of *Mirabilis jalapa* defensins, MjAMP-1 was N-terminally blocked and MjAMP-2 was not blocked. The remainder of the protein differed in only three positions (Cammue et al., 1992).

Most of the plant defensins isolated to date are antifungal. The only known exceptions are plant defensins isolated from some members of Poaceae including Siα1, Siα2 and Siα3 from wheat, barley and sorghum seeds, respectively. These defensins do not inhibit fungal growth but have been reported to inhibit α-amylases from insects and humans (Bloch and Richardson, 1991; Osborn et al., 1995). Most antifungal plant defensins such Ah-AMP1, Ct-AMP1, Dm-AMP1, Hs-AMP1, and Rs-AFP2 do not inhibit α-amylase (Osborn et al., 1995; Terras et al., 1992).

The majority of the plant defensins are very potent antifungal agents and exhibit a broad range of antifungal activity (Table II-9) at very low concentrations (Broekaert et al., 1996; Cammue et al., 1992; Osborn et al., 1995; Terras et al., 1995). At the same time, antifungal potency of each defensin varies depending on the fungus tested. For example MjAMP-1 and MjAMP-2 exhibited antifungal activity against 13 different plant pathogenic fungi. The amount of these defensins required to inhibit fungal growth by 50% (IC 50) varied from 0.5 μg to 300 μg /ml in a 48 h assay depending on the test organism (Cammue et al., 1992). Radish defensins RsAMP-1 and RsAMP-2 inhibited growth of 20 different plant pathogenic fungi. The IC-50 value for these two defensins varied from 0.4 μg to 100 μg/ml. Similar results were obtained with AcAMP-1 and AcAMP-2 from *Amaranthus caudatus* and CaAMP-1 from *Capsicum*. AcAMP-1 and AcAMP-2 were active against 13 different plant pathogenic fungi with IC50 values between 0.8 and 20 μg/ml. CaAMP-1 inhibited growth of 18 different plant pathogens with IC50 values between 1 and 500μg/ml depending on the fungus tested.

Table II-9: Broad Host Range Antifungal Activity of Several Defensins.

Defensins	**Plant source**	**Defensin sensitive Fungi**	**Reference**
Rs-AFP1	*Raphanus sativus.*	20	Terras et al., 1992
Rs-AFP2	*Raphanus sativus.*	20	Terras et al., 1992
Ac-AMP1	*Amaranthus caudatus*	14	Broekaert et al., 1996
Ac-AMP2	*Amaranthus caudatus*	14	Broekaert et al., 1996
Ca-AMP1	*Capsicum annum*	19	Broekaert et al., 1996
Bm-AMP1	*Brizza maxima*	8	Broekaert et al., 1996
Dm-AMP1 Hs-AMP1 Ah-AMP1 Ct-AMP1	*Dahlia merckii;* *Heuchera sanguinea;* *Aesculus hippocastanum;* *Clitoria ternatea*	8	Osborn et al., 1995
Mj-AMP1	*Mirabilis jalapa*	13	Cammue et al., 1992

The antifungal activity of plant defensins is generally resistant to heat, extreme pH and proteases. For example, antifungal activity of *Amaranthus* defensins was not affected by heat treatment at up to 100°C for 10 min, exposure to acidic (pH 2) or alkaline pH (pH 11) conditions or treatment with proteases including proteinase K, pronase E, chymotrypsin and trypsin (Broekaert et al., 1996).

Despite the similarity in amino acid sequence and tertiary structure, the plant defensins show marked differences in their antifungal activity including differences in their unit activity and range of pathogens affected. Based on their morphological effect on fungi, the plant defensins are divided into two groups. Group I the morphogenic defensins, cause hyphae to swell, reduce hyphal elongation and increase hyphal-branching. Examples of morphogenic plant defensins include RsAMP-1 and RsAMP-2 from radish and Hs-AFP1 from *Heuchera senguinea*. Group II, the non-morphogenic defensins, slow hyphal growth without any marked change in hyphal morphology. Examples of non-morphogenic defensins include DmAMP-1 from Dahlia, CtAMP-1 from *Clitoria* and AhAMP-1 from horse chestnut seeds.

The efficacy of plant defensins varies depending on the protein's pI-value. Generally, the more basic proteins exhibit higher antifungal activity (Broekaert et al., 1996; Cammue et al., 1992; Terras et al., 1995). In the case of the defensins from *Mirabilis*, *Raphanus*, *Capsicum*, *Amaranthus* and *Briza*, the more basic isoforms are more active showing 2 to 30-fold higher activity than the less basic forms. The antifungal activity of plant defensins is affected by the ionic composition of the growth media. Both morphogenic and non-morphogenic defensins have reduced antifungal activity in the presence of monovalent and divalent cations, especially calcium (Broekaert et al., 1996; Terras et al., 1995). This phenomenon has been observed with insect and mammalian defensins (Cociancich et al., 1993; Lehrer et al., 1993).

Thevissen reported that Rs-AFP2 from radish and DmAMP-1 from dahlia cause rapid ion fluxes when added to fungal hyphae i. e. Ca^{+2} uptake, K ion efflux and pH of the medium all increase (Thevissen et al., 1996). The cation sensitivity of radish defensins varies depending on the fungus tested. Terras proposed that the antagonistic effect of cations is not through direct interaction with the defensin causing conformational changes in the proteins (Terras et al., 1995). They concluded that the fungus interacts directly with

the cation and thereby acquires protection from the effects of these toxic proteins.

Branching of fungal hyphae is regulated by specific Ca^{+2} channels (Robson et al., 1991). Rs-AFPs from radish are morphogenic defensins and cause hyperbranching suggesting that these defensins may interfere with Ca^{+2} signaling (Terras et al., 1995). The specific relationship between pI, fungal calcium ion uptake and antifungal activity was clearly demonstrated by De Samblanx and co-workers (De Samblanx et al., 1997). In mutational studies with Rs-AFP2, De Samblanx et al. demonstrated that by replacing the amino acid residues in non-conserved positions with arginine, the antifungal activity of the substituted analogs Rs-AFP2 (G9R) and Rs-AFP2 (V39R) was enhanced. These more basic variants caused increased Ca^{+2} uptake while another variant, Rs-AFP2 (Y38G), that was devoid of antifungal activity was unable to stimulate Ca uptake in the fungus tested. The antifungal activity of the more positively charged variants was less susceptible to the presence of cation in the media relative to the wild type Rs-AFP2.

Two highly hydrophobic and adjacent sites in Rs-AFP2 were also critical for antifungal activity. These two sites may be part of a putative signal receptor. Alternatively, the two sites may represent two independent

binding sites on each of two receptor sites (Terras et al., 1995). Rs-AFP2 cDNA was introduced into and constitutively expressed in transgenic tobacco (Terras et al., 1995). Transgenic plants expressing Rs-AFP2 defensin exhibited a 7-fold reduction in lesion size after infection with *Alternaria longipes*.

Although most plant defensins isolated to date are from seeds, it appears that these proteins are constitutively expressed at lower concentrations in healthy, vegetative tissues (Broekaert et al., 1995; Moreno et al., 1994; Terras et al., 1995). The levels of these leaf defensins increase rapidly upon infection. For example, radish Rs-AFP counterparts in the leaf were strongly and systemically induced upon fungal infection or treatment with mercuric chloride (Terras et al., 1995). Plant defensins are produced in the peripheral layer of seeds or vegetative tissues. Thus, induction of defensin genes in vegetative tissue may provide protection against pathogens that reside in the outer tissues of leaves (Moreno et al., 1994; Terras et al., 1995). According to Terras and co-workers, these proteins together with different PR-proteins such as chitinases and glucanases may prevent the development of fungal diseases in vegetative tissue (Terras et al., 1995). The same is true for the seed defensins that are present extracellularly and

are located in the cell wall and outer cell layers lining different seed tissues. Seed defensins released during germination may protect the germinating seedling from soil borne pathogens.

Thionins

Thionins are low molecular weight (approximately 5 kDa) proteins found in roots, stems, leaves, flowers and seeds of a number of plants (Florack and Stiekema, 1994; Gu et al., 1992). Depending on the net charge, number of amino acids residues, disulfide bonds, plant species and tissue source, thionins are divided into four different types (Florack and Stiekema, 1994; Garcia-Olmedo et al., 1989). Type 1 thionins are present in members of the Poaceae. These thionins are present primarily in endosperm tissue. They are highly basic and consist of 45 amino acids and 4 disulfide bonds. Type 2 thionins are found in the leaves of barley and in the leaves and nuts of the parasitic plant *Pyrularia pubera* (Bohlmann et al., 1988). Type 2 thionins are slightly less basic than type 1 thionins. These thionins are 46-47 amino acids long and have 4 disulfide bonds. Leaves and stems of *Phoradendron* species contain type 3 thionins (Samuelsson and Pettersson, 1971; Schrader and Apel, 1993). Type 3 thionins are 45-46 amino acids long, have 3-4 disulfide bonds and are as basic as type 2 thionins. Type 4 thionins are

found in seeds of *Abyssinian* cabbage. These thionins are 46 amino acids long, have three disulfide bonds and are neutral (Van Eaten et al., 1965). All four types of thionins are highly homologous at the amino acid level. The position and number of disulfide bonds are highly conserved and all thionins have a tyrosine residue at position 13.

Analysis of thionin cDNA sequences reveals that thionins are synthesized as preproproteins that undergo post-translational modification. The precursors contain three distinct domains. The first is an N-terminal signal peptide that is involved in the transfer of the protein into the lumen of the ER. Adjacent to the N-terminal signal peptide is the mature thionin. The last is the C-terminal domain. According to Florack et al. (1994), the C-terminal domain is involved in the transport of thionins through membranes.

Thionins are toxic to fungi (Molina et al., 1993; Terras et al., 1993; Terras et al., 1996). The three dimensional structure of the protein reveals that thionins consist of an outer part which is mostly hydrophobic and an inner part that is mostly hydrophilic. The outer hydrophobic surface of the positively charged thionins presumably interacts with the negatively charged polar tail groups of the phospholipid of the host membrane. This anchors the protein so that the hydrophilic core can extend into the aqueous phase of the cytoplasm (Teeter et al., 1990; Wada et al., 1982).

Non-specific lipid transfer protein:

Non-specific lipid transfer proteins (nsLTPs) are present in both monocots and dicots (Arondel and Kader, 1990; Bernard et al., 1991). The nsLTP's are basic polypeptides containing 90 to 93 amino acid residues including eight cysteine residues. In *in vitro* experiments, these proteins transfer phospholipids and glycoproteins between organelle membranes, liposomes and mitochondria (Arondel and Kader, 1990; Kader, 1993). The nsLTP precursors contain signal peptides. They may be secreted or bound to the cell wall (Bernard et al., 1991; Thoma et al., 1993). The nsLTPs are the major proteins in the surface wax of broccoli and are involved in cuticle formation. The antifungal properties of nsLTPs purified from radish, onion and sugar beet has been reported (Cammue et al., 1995; Nielsen et al., 1996; Terras et al., 1992). Radish and onion nsLTP are active at very low concentration (below 10μg/ml) and exhibit a broad range of antifungal activity.

The antifungal activity of Ace-AMP (nsLTP from *Allium cepa*) was weakly affected by the presence of different cations. On the other hand, antifungal activity of radish nsLTP is affected by the presence of even low concentrations (1 mM) of calcium ion in the growth medium (Terras et al.,

1992). Although Ace-AMP exhibits a high degree of homology to other nsLTPs, this protein was unable to transfer phospholipids from liposomes to mitochondria (Cammue et al., 1995). The protein structure predicted from the sequence of the cDNA revealed that Ace-AMP has a 12 residue C-terminal peptide that was absent from the mature protein (Cammue et al., 1995). It is not clear whether the absence of this peptide accounts for differences in activity.

Not all non-specific lipid transfer proteins have antifungal activity. Some isomers of nsLTPs may be involved in defense while others deposit extracellular lipids such as cutin monomers. According to Terras and associates some nsLTPs acquire defensive roles after their deposition on the cell wall along with the cutin they transfer (Terras et al., 1995). Thus, it is possible that the direct antifungal activity of nsLTP's is a secondary function.

Two antifungal proteins with homology to nsLTPs and with very strong activity against *Cercospora beticola* were isolated from the intercellular fluids of sugar beet (Nielsen et al., 1996). A cDNA encoding a similar isoform of these sugar beet proteins has been isolated and characterized (Nielsen et al., 1996). The expression of these proteins was not induced upon infection with *C. beticola*. Gene expression in the

uninfected plant was developmentally regulated even though constitutive accumulation of mRNA was observed. *C. beticola* was restricted to the extracellular environment during the entire infection process. Sugar beet nsLTPs, isolated from the apoplastic compartment of these plants, were very effective inhibitors of *C. beticola* growth. The presence of nsLTPs in the intercellular space apparently helps to prevent *C. beticola* infection.

Ribosome-inactivating proteins:

Plant ribosome-inactivating proteins (RIPs) catalytically cleave N-glycosidic bonds of a single adenine residue of the large ribosomal RNA. Altered rRNA can not bind to the elongation factor 2 and, consequently, protein synthesis is inhibited (Roberts and Selitrennikoff, 1986; Stirpe et al., 1992). RIPs are found in a wide variety of monocots and dicots. RIPs were first known for their antiviral activities (Stirpe et al., 1992). RIPs purified and characterized so far have been divided into two different groups (Stirpe et al., 1992). Type 1 RIPs consist of a single polypeptide chain with RNA glycosidase activity. Type 2 RIPs are composed of two polypeptide chains, an A chain with RNA glycosidase activity and a B subunit containing a galactose-specific lectin domain. The B chain of type 2 RIPs binds to the cell surface and promotes uptake of the A-chain into the cell. Once the A-

chain enters the cell, it inhibits protein synthesis by modification of 28S ribosomal RNA. Type 1 RIPs lacking the ability to bind to cells are not cytotoxic.

Type 2 RIPs are extremely potent cytotoxins. Examples of type 2 RIPs include ricin from *Ricinus communis*, abrin from *Abrus precatorious* and viscacin from mistletoe. Type 2 RIPs are 1000-times more active on mammalian ribosomes, while type 1 RIPs have a broad spectrum specificity and cleave rRNA from both eukaryotic and prokaryotic organisms (Prestle et al., 1992; Zoubenko et al., 1997). Type I RIPs are more common than type II RIPs and a number of type I RIPs have been isolated from representatives of different plant families. Examples of type I RIPs include saporin from soapwart, asparin from asparagus, pokeweed antiviral protein (PAP), Mirabilis antiviral protein (MAP), tritin from wheat, barley RIP, corn RIP and amaranthin from *Amaranthus viridis* (Chaudhry et al., 1994; Habuka et al., 1993; Hey et al., 1995; Kataoka et al., 1993; Kwon et al., 1997; Stirpe et al., 1983; Watanabe et al., 1997).

Although a 30-kDa type I RIP from barley seed showed antifungal activity against *Tricoderma resii* and *F. sporotrichoides*, the activity was much lower than chitinases and glucanases isolated from the same plant (Leah et al., 1991). Although barley chitinase (CHI 26) and glucanase (BGL

32), showed better antifungal activity than RIP 30 when tested alone, the strongest antifungal activity was observed when these three proteins were combined. According to Leah et al. (1991), the activity of RIP was enhanced by chitinase and glucanase dependent hydrolysis of cell wall components. Once the cell wall constituents are removed, RIP may enter the cytoplasm and reach its target site.

In principle, type 2 RIPs should be toxic to fungi. However, the thick fungal cell wall may prevent penetration and binding and, thereby, reduce or eliminate the toxicity of these proteins. To this date, there is no evidence for the direct antifungal effect of type 2 RIPS. A barley RIP gene under the control of a wound-inducible promoter was introduced into tobacco (Logemann et al., 1992). The transgenic R1 progeny exhibited enhanced resistance to *R. solani* (Logemann et al., 1992). Constitutive co-expression of barley endosperm RIP and barley chitinase II exhibited significant improvements in resistance to *R. solani* in transgenic tobacco (Jach et al., 1995). Similarly, expression of pokeweed antiviral protein (PAP) in transgenic tobacco activates multiple plant defense pathways and confers resistance to *R. solani*. The resistant plants expressed high levels of both class I and class II isoforms of PR proteins (Zoubenko et al., 1997). Thus, RIPs may have an indirect role in the regulation of plant gene expression.

Lectins

Plant lectins are a unique class of proteins with the ability to bind to carbohydrates. In many cases, these proteins agglutinate red blood cells. Concanavalin A (Con A) was the first lectin to be isolated. This lectin was isolated from *Canavalia ensiformis* (jack bean) in 1936 (Sumner and Howell, 1936). Since then, over 100 lectins have been purified from species of diverse plant families [for detailed review, see (Lis and Sharon, 1986; Lis and Sharon, 1981)]. Plant lectins occur in seeds and vegetative tissue such as roots, leaves, rhizomes, latex, bark and the flowers (Peumans and Van Damme, 1995). Depending on the specificity of carbohydrate binding, plant lectins are divided into five major groups (Table II-10). The native molecular weight of lectins varies from 4.5 kDa (hevein) to 265 kDa (lima bean lectin). Lectins may exist as monomers or homo or hetero-multimers.

Table II-10: Monosaccharide Specificity of Some Lectins.

Group	Sugar	Lectin
I	Mannose Glucose	Concanavalin A Fava bean Lentil Pea
II	N-acetylglucosamine	Wheat germ *Ulex europeus*
III	N-acetylgalactosamine	Soybean Lima bean *Dolichos biflora*
IV	Galactose	Peanut *Ricinus communis*
V	L-fucose	*Ulex europeus* *Lotus tetragonolobus*

Lectins such as Conconavalin A tend to aggregate depending on conditions. For example, at pH below 6.0, Con A exists as a monomer (51 kDa) but aggregates to form a dimer at physiological pH (102 kDa). Plant lectins are stable over a wide range of pH. They are frequently heat resistant and are not destroyed by animal or insect gut proteases. With few exceptions, most lectins contain metal ions and in some instances, the metal ion is necessary for carbohydrate binding.

Because of the widespread distribution of lectins in the plant kingdom and the abundance of lectins in many plants, it is possible that lectins play important physiological roles in plants. Lectins may function in seed maturation and germination, maintenance of seed dormancy and as storage proteins (Etzler, 1985; Peumans et al., 1983; Shakirova et al., 1994). Lectins are capable of exerting a variety of biological effects on human, animal, insect, fungal and bacterial cells and viruses. Not all the biological effects are observed with all lectins but different lectins are responsible for different biological activities. This review will be restricted to a discussion of the antifungal properties of plant lectins.

Because some lectins are able to bind to chitin, researchers speculated that lectins have antifungal properties. The first observation of antifungal lectins was reported by Mirelman and co-workers (Mirelman et al., 1975).

According to this report, wheat-germ agglutinin (WGA) inhibits spore germination and hyphal growth of *T. viride*. However, the antifungal role of lectins was seriously questioned when Schlumbam and associates (Schlumbum et al., 1986) demonstrated that highly purified WGA did not have any antifungal activity. Subsequently, it was shown that the original preparations of WGA were contaminated with chitinase. Later Broekaert and co-workers demonstrated that chitinase-free potato lectin did not exhibit any antifungal activity (Broekaert et al., 1989).

Conclusive evidence for the existence of antifungal lectins was obtained by Broekaert et al. (1989). This research group demonstrated conclusively the antifungal activity of chitinase-free nettle lectin isolated from nettle rhizomes. Nettle lectin was more active than chitinase against *Botrytis cineria* whereas chitinase was more active against *T. hamatum*. Nettle lectin (UDA), a small (8.5 kDa) monomeric lectin is not blood group specific. Nettle lectins do not bind to chitin monomers effectively and do not agglutinate blood cells very well. Another antifungal lectin, hevein, was isolated from *Hevea brasiliensis* latex. Hevein is a small monomeric lectin (4.7 kDa) with strong homology to the amino acid sequence of nettle lectin. UDA and hevein are the only two monomeric lectins isolated so far. Both of these proteins are cysteine rich and heat resistant. These proteins are not

denatured by boiling for 10 min (Broekaert et al., 1989; Parijs et al., 1991). Chitinase-free hevein exhibited antifungal activity against eight different fungi including plant pathogenic fungi such as *F. oxysporum*, *F. culmorum* and *Pyrecularia oryzeae*. Expression of lectin genes can be triggered in vegetative tissue in response to stress. Wounding of potato tuber resulted in increased accumulation of lectins (Miller et al., 1992).

It is not clear why some chitin-binding lectins such as nettle lectin and hevein exhibit antifungal properties, while others such as WGA or potato lectin do not. Both UDA and hevein are very small proteins whereas WGA (86 kDa) or potato lectin (100 kDa) are not. It is possible that the small size of the antifungal lectins is related to the antifungal activity. According to Parijs, these small antifungal lectins may penetrate through the fungal cell wall and reach the plasma membrane where they inhibit synthesis of chitin or interfere with chitin deposition (Parijs et al., 1991). Hevein and UDA show the same morphological affect on fungi. Both of these antifungal lectins inhibit fungal growth by producing thick hyphae with buds, in contrast to chitinase that causes lysis of the hyphal tip.

Chapter III : Evaluation of Tropical Plant Extracts for Antifungal Activity

INTRODUCTION

Tropical rainforests occupy approximately 7% of the earth's land surface, yet more plants and animals exist in these forests than in all of the world's other ecosystems combined (Wilson, 1991). There are a number of layers in a tropical rainforest and different life forms have evolved to survive in these layers resulting in a tremendous amount of biodiversity (Erwin, 1991). For example, 151 different tree species are found in a 100 sq. meter section of Barro Colorado Island. In contrast, there are only 30 different tree species in a 10,000 sq. meter area in the Appalachian mountains. In a one hectare surveyed plot of Peruvian Amazon, 300 different tree species have been identified (Wilson, 1991).

Not only is there a tremendous amount of biodiversity among plant species in the tropical rainforest, but there is also a tremendous diversity of animals, insects, fungi and microbes. E.B. Wilson identified 43 different ant species in one legume tree located in Tombapata Reserve in Peru (Wilson, 1987). It is common to find thousands of species of beetles and other insects

in one sq. kilometer of forest in Central and South America (Erwin, 1991; Wilson, 1991). Because of the enormous species diversity in the tropical rainforest, there is extreme competition between species for survival. In order to protect themselves from a diverse range of pests and pathogens, tropical rainforest plants have evolved an arsenal of defense mechanisms.

Fungi are considered as one of the major pathogens of plants. The moist, warm conditions and high organic content of tropical forests provide a perfect environment for fungal growth and propagation. Therefore, it is only reasonable that tropical plants produce fungicidal compounds to protect themselves from these pathogens (Robinson, 1991). Various pesticidal compounds have been isolated from a number of tropical plants. Most of these compounds have insecticidal properties and have been characterized as low molecular weight secondary metabolites. Although tropical plants are a rich source of fungicidal compounds, the search for antifungal compounds in tropical plants remains unexplored.

To discover antifungal activity from this rich natural source, more then 100 tropical rainforest species were collected and screened for antifungal activity. Crude extracts from these species were tested for antifungal activity before and after dialysis. Dialysis was done to separate

the low molecular weight (less then 3,500 Da) from high molecular weight (greater then 3,500 Da) antifungal activities. Emphasis was given to those activities retained upon dialysis on the assumption that the high molecular weight activity could be a protein. Many antifungal proteins have been purified and characterized but very few of them are from tropical rainforest plants. Most of the purified and characterized antifungal proteins cited in the literature are from spices, agricultural crops or common flowering plants.

Our objective was to discover, isolate and characterize antifungal proteins from tropical plants. In this chapter, we report on the results of our initial screen. After the initial screen, several extracts exhibited antifungal activities that were retained after dialysis. These findings provided a preliminary indication that these activities were due to macromolecules, possibly proteins, and not low molecular weight secondary metabolites. The purification and characterization of several of these activities is presented in subsequent chapters.

MATERIALS AND METHODS

Source of Plant Material

All plant materials used for the antifungal screen were collected from the La Selva Biological Station, Costa Rica. Permits for plant collection and exportation were obtained from the appropriate governmental authorities in Costa Rica. Importation permits required to bring biological materials into the U.S. were obtained from the Animal Plant Health Inspection Service (APHIS), of the U.S. Department of Agriculture. Plant samples were collected based on field observations and ethnobotanical information. In a few cases, plant materials were collected by an external contractor. After collection, plant materials were processed in the laboratory at La Selva Biological Station. Biological materials were separated into seeds, roots, leaves, fruits etc., labeled and stored in a –20°C freezer or on dry ice. In general, voucher specimens were prepared for each collection and maintained for identification and future reference. The frozen plant tissues and voucher specimens were imported into the United States. In the laboratory at the University of Oklahoma the plant tissues were stored at –40°C.

Extraction of Plant Materials

Plant materials (seeds, fruits, leaves, roots, stems) were extracted in 10 mM Tris-HCl (pH 8.0) containing 0.2 g of insoluble PVP (polyvinylpolypyrrolidone; Sigma Chem. Co, St. Louis, Mo; Cat. # P6755) for each g of plant tissue. Tris-HCl buffers were prepared using a mixture of Tris-free base and Tris-HCl. Plant tissue was homogenized in a polytron homogenizer and the homogenate was filtered through a double layer of Miracloth. The filtrate was centrifuged at 15,000 x g for 15 min in a Sorvall SS34 rotor. The supernatant fluid was collected and the pellet containing debris and insoluble PVP was discarded. This clarified supernatant fluid, referred to as the crude extract, was tested for antifungal activity.

Dialysis

To remove soluble, low-molecular-weight materials from the crude extract, a volume of each crude extract was dialyzed extensively against 10 mM Tris-HCl (pH 8.0) using a 3,500 molecular weight cut off dialysis membrane (Spectra/Por). Dialysis was routinely carried out in 4 L beakers and the

dialysis buffer was changed at least three times over a 24-48 h period. After dialysis, the crude extract (referred to as dialyzed extract) was stored frozen at –20°C.

Source of Fungal Pathogens

Cultures of *Fusarium chlamydosporum*, *Aspergillus flavus* and *Aspergillus parasiticus* were used for the initial antifungal screen. The antifungal activity of all extracts was evaluated on the basis of their antifungal activity against *F. chlamydosporum*. *A. flavus* and *A. parasiticus* were used only in selected assays. Cultures of *A. parasiticus* were obtained from the Department of Botany and Microbiology culture collection at the University of Oklahoma. *A. flavus* (ATCC # 22548) and *F. chlamydosporum* (ATCC # 22187) cultures were obtained from the American Type Culture Collection, Waldorf, MD. Working cultures of *A. flavus* and *A. parasiticus* were maintained at room temperature on full strength potato dextrose agar (PDA; Difco # 0013-17-6) and *F. chlamydosporum* was grown at room temperature on half strength PDA. All three fungi were kept at room temperature for ten days or until the mycelial growth covered three fourths of the plate. At that

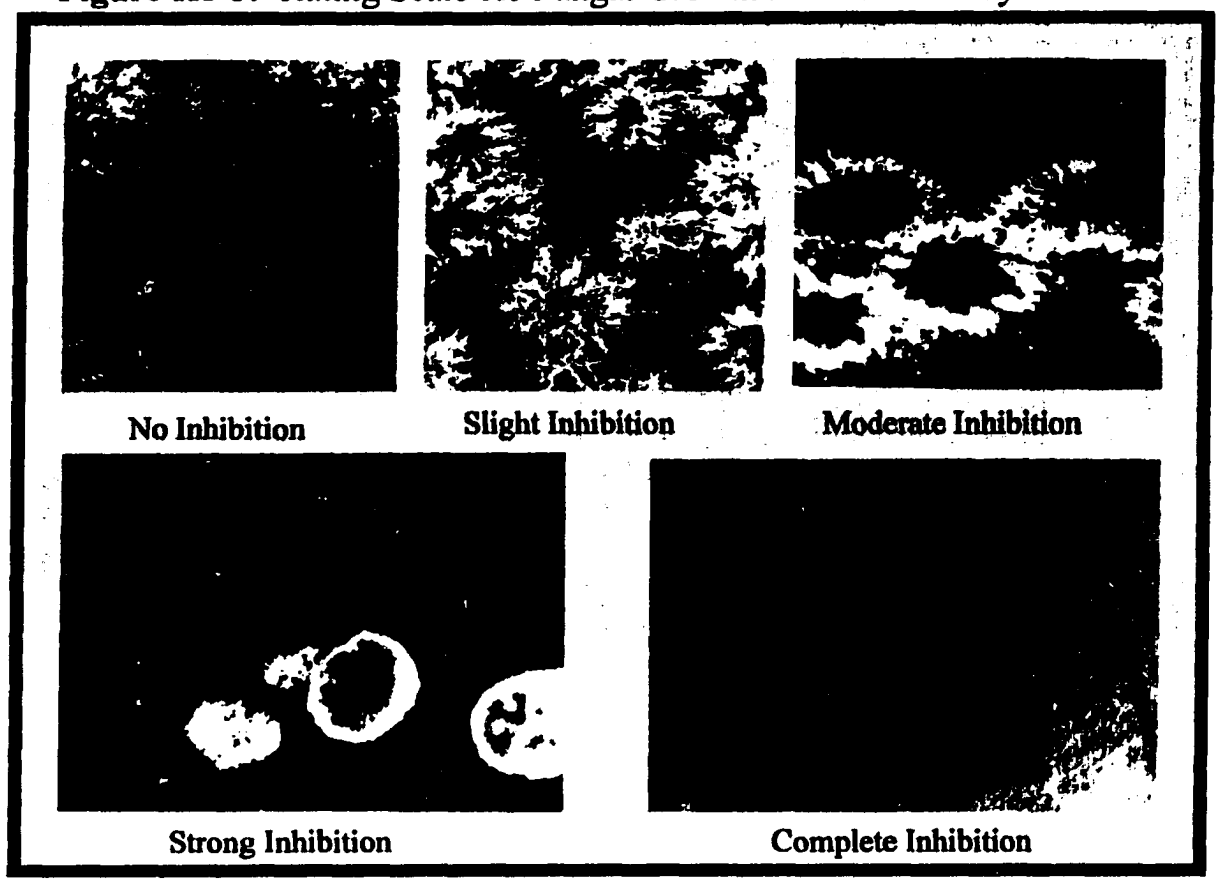
point, plates were kept at 4°C. For long term preservation, conidia of *A. flavus* and *F. chlamydosporum* were preserved on sterile silica gel at 4°C according to the method of Windels (1992).

Antifungal Bioassay

The antifungal assay used to detect antifungal activity in plant extracts was originally developed by Duvick and associates (Duvick et al., 1992). Conidia of all three fungi were collected for antifungal bioassay by scraping the colony with a sterile loop and suspending the conidia in sterile water containing 0.01% Tween 20. Conidia from this stock solution were diluted with synthetic culture medium to a final concentration of approximately 290 conidia/90 µl of growth medium. The latter contained 0.037 g NaCl, 0.0625 g $MgSo_4$ $7H_20$, 0.25 g $CaN0_3$, 2.5 g glucose, 0.25 g yeast extract, and 0.125 g casein enzyme hydrolysate in one liter of 7.5 mM sodium phosphate buffer, pH 7.0. Ninety µl of the culture medium containing conidia was added to each well of a 96-well, U-bottom microtiter plate. Ten µl of crude extract or crude dialyzed extracts was added to each well. Four replicates (individual wells) were used for each sample. Sample buffer was used as a control. The microtiter plate was covered with parafilm and incubated in the dark at 25°C for 48 h. Conidial germination and fungal growth were

observed after 48 h using an inverted microscope. A rating scale of 0 to 4 was used to evaluate inhibition of fungal growth (Figure III-1). The ratings were based on the relative growth of fungi in the buffer control. A rating of zero indicated no inhibition of fungal growth and a rating of four was given in the case of complete inhibition of fungal growth. Intermediate values were assigned to distinguish between ratings when possible. Values from the four replicates were averaged.

Figure III-1: Rating Scale for Fungal Growth Inhibition Assay.



RESULTS

Results of extracts from PCN 1 to 40

Out of the first 41 extracts, 38 extracts exhibited moderate to complete inhibition of fungal growth (rating of 2 to 4) before dialysis (Figure III-2 and III-3). Only five extracts (2L, 5L, 10L, 15F and 34LT) had ratings of 2 or lower. Among the 38 crude extracts showing moderate to strong antifungal activity, eleven inhibited *F. chlamydosporum* growth moderately (rating 2 to less than 3), sixteen crude extracts exhibited very strong inhibition (3-3.6) and eleven extracts inhibited fungal growth completely.

A selected group of the first 40 extracts (1S, 7L, 8S, 14L, 21F, 23L, 33L, 34LT, 35LT and 40L) were exhaustively dialyzed (3,500 MWCO) and assayed for antifungal activity. Dialyzed 40L and 1S completely inhibited conidial germination of *F. chlamydosporum* (Figure III-4). Loss of substantial activity was observed in extracts 7L, 8S, 14L, 21F, 23L, 33L and 35LT. All of these extracts exhibited complete to very strong inhibition before dialysis but after dialysis, these extracts exhibited only moderate to slight (rating less than 1) inhibition of fungal growth in the bioassay.

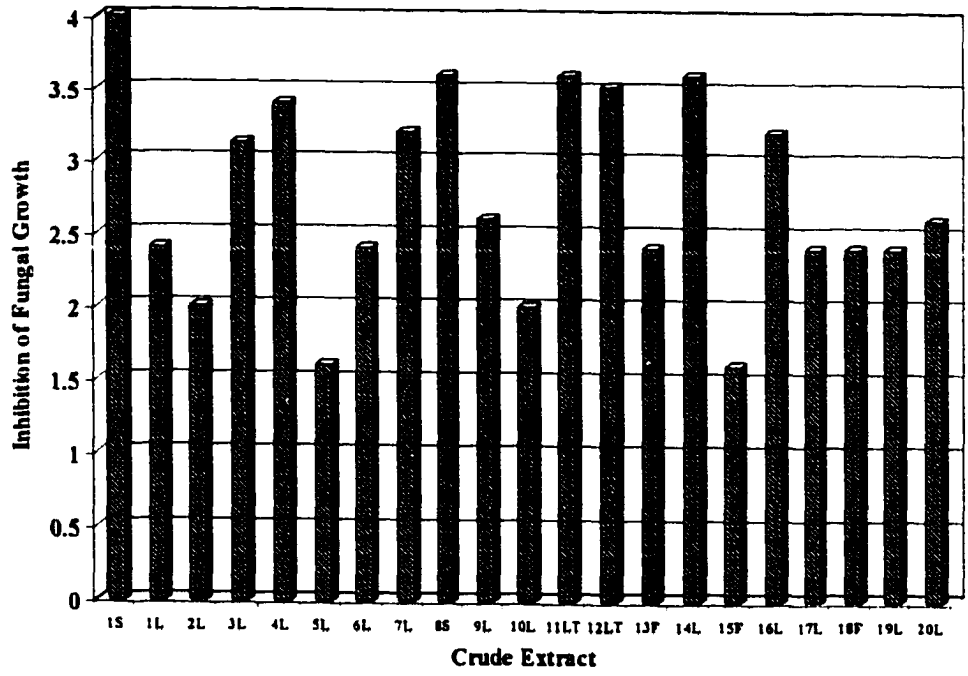


Figure III-2: Evaluation of Antifungal Activity of Crude Extracts from Plant Collection Number (PCN) 1-20. Antifungal activity was measured using the standard assay with *F. chlamydosporum*. Rating of 4 = complete inhibition of conidial germination and hyphal growth, rating of 0 = no inhibition of fungal growth. (L = leaf, S = seed, F = fruit, LT = latex).

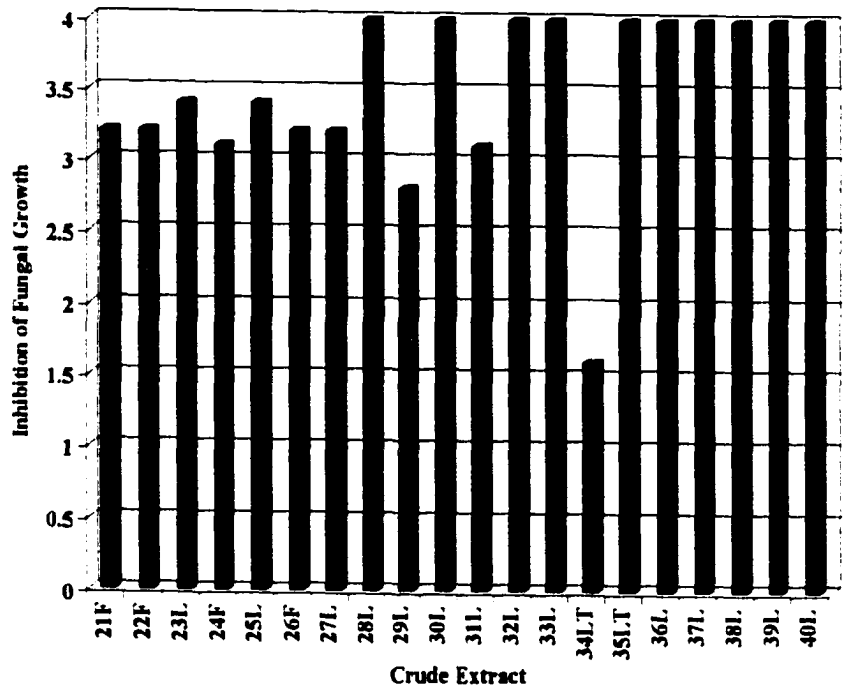


Figure III-3: Evaluation of Antifungal Activity of Crude Extracts of PCN 21-40. Antifungal activity was measured using the standard assay with *F. chlamydosporum*. Rating of 4 = complete inhibition of conidial germination and hyphal growth. (L = leaf, F = fruit, LT = latex).

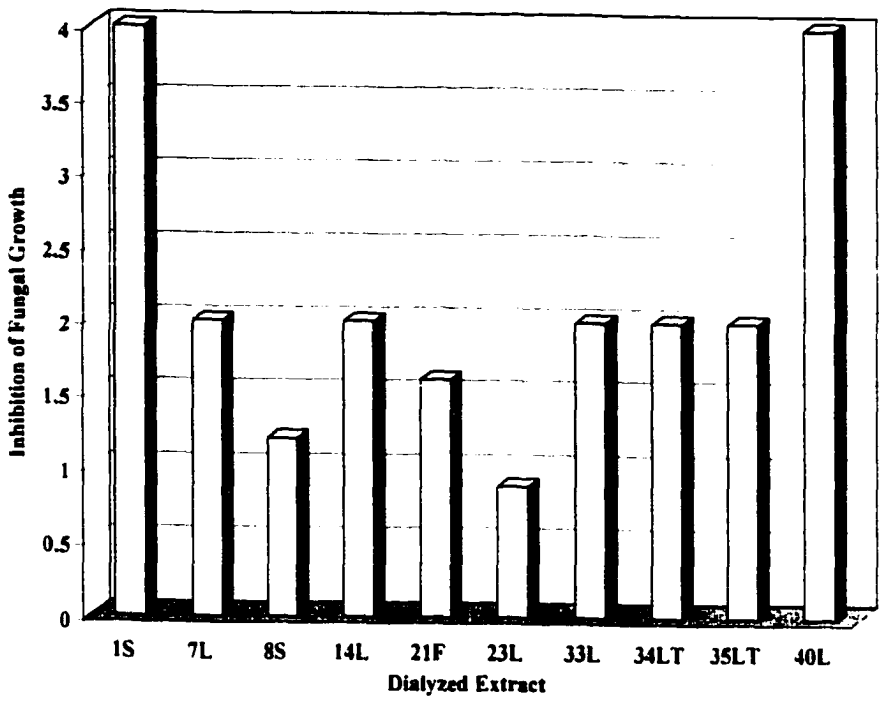


Figure III-4: Evaluation of Antifungal Activity of Selected Dialyzed Extracts. Antifungal activity was measured using the standard assay with *F. chlamydosporum*. Rating of 4 = complete inhibition of conidial germination and fungal growth, rating of 0 = no inhibition of fungal growth. (L = leaf, F = fruit, S = seed, LT = latex).

Results of extracts from PCN 41 to 100

Seven crude extracts from the group 41 to 100 did not exhibit any antifungal activity (Figure III-5, III-6 and III-7) against *F. chlamydosporum*, while 42 showed slight inhibition of fungal growth (rating below 2) in the bioassay. Three crude extracts exhibited moderate antifungal activity. Very strong inhibition of fungal growth was observed in extracts 47F, 48L and 69L. Crude extracts, 43S, 65F, 70L and 98F exhibited complete inhibition of conidial germination.

Fifty-two of the 60 extracts from this group exhibited antifungal activity before dialysis, but majority of these extracts lost activity after dialysis (Figure III-5, III-6 and III-7). Ten extracts out of the 14 extracts that retained antifungal activity after dialysis exhibited only a slight inhibition (rating of 1 or below) of *F. chlamydosporum* growth. Dialyzed extract of 100S showed moderate inhibition and very strong inhibition of fungal growth was observed in the dialyzed extract of 48L. The antifungal activity was completely retained by extract 98F. This dialyzed extract completely inhibited conidial germination of *F. chlamydosporum*. Extract 98F was the only extract among this group that completely inhibited fungal growth before and after dialysis.

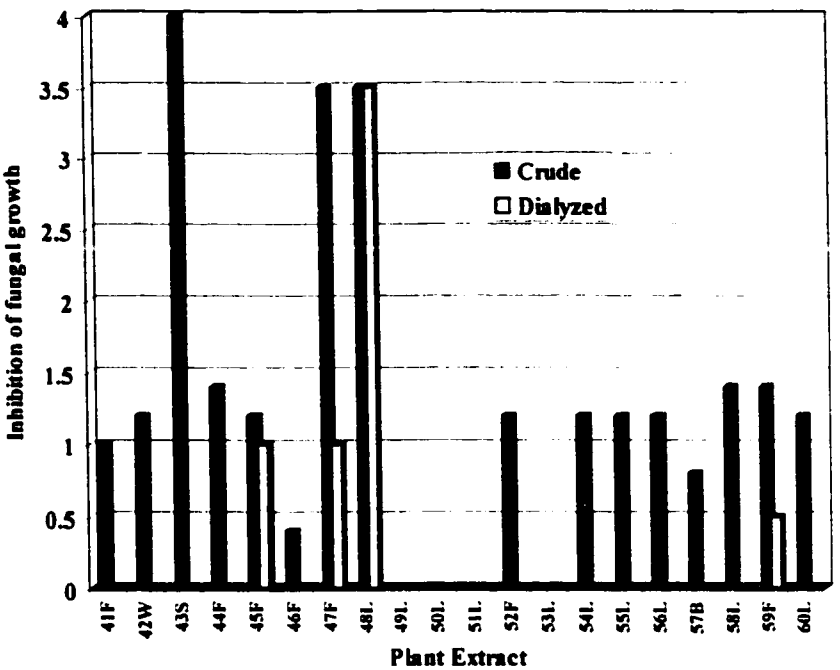


Figure III-5: Evaluation of Antifungal Activity of Crude and Dialyzed Extracts of PCN 41 to 60. Antifungal activity was measured using the standard assay with *F. chlamydosporum*. Rating of 4 = complete inhibition of conidial germination and hyphal growth, rating of 0 = no inhibition of fungal growth. (L = leaf, S = seed, F = fruit, W = whole plant).

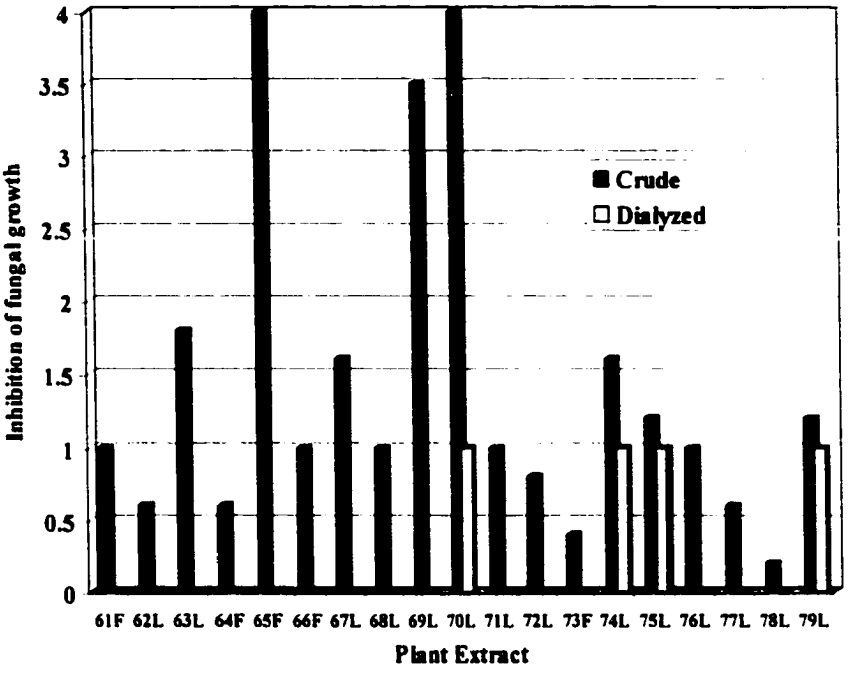


Figure III-6: Evaluation of Antifungal Activity of Crude and Dialyzed Extracts of PCN 61 to 79. Antifungal activity was measured using the standard assay with *F. chlamydosporum*. Rating of 4 = complete inhibition of conidial germination and hyphal growth, rating of 0 = no inhibition of fungal growth. (L = leaf, F = fruit).

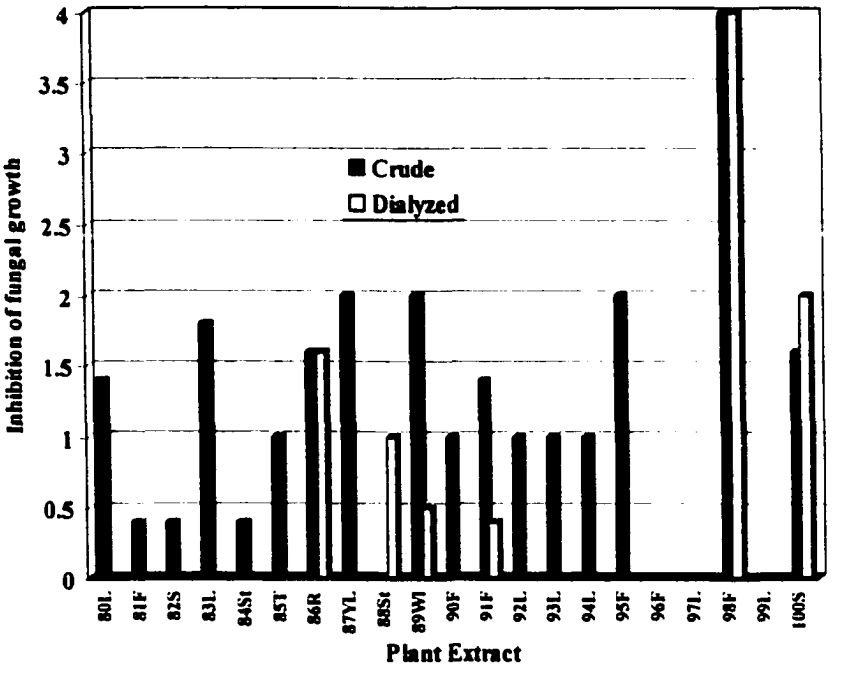


Figure III-7: Evaluation of Antifungal Activity of Crude and Dialyzed Extracts of PCN 80 to 100. Antifungal activity was measured using the standard assay with *F. chlamydosporum*. Rating of 4 = complete inhibition of conidial germination and hyphal growth, rating of 0 = no inhibition of fungal growth. (L = leaf, F = fruit, St = stem, T = tuber, YL = young leaf, W = whole plant).

Results of extracts from PCN 101 to 158

Antifungal activity against *F. chlamydosporum* was observed in eighteen crude extracts from extracts 101 to 158 (Figure III-8, III-9 and III-10). Out of these eighteen crude extracts, three extracts exhibited only slight inhibition and 14 extracts exhibited moderate inhibition (rating 1.5 to 2.6) of *F. chlamydosporum* mycelial growth. Extract 157L was the only crude extract from PCN 101 to 158 that showed complete inhibition of fungal growth.

After dialysis there were sixteen extracts out of this group, that exhibited antifungal activity against *F. chlamydosporum* (Figure III-8, III-9 and III-10). Out of these sixteen, nine extracts (101L, 107L, 115L, 115S, 119L, 122L, 129 W, 140L, 144L) inhibited fungal growth after dialysis but not before dialysis. Extract 140L completely inhibited *F. chlamydosporum* growth. Extract 115S inhibited *F. chlamydosporum* growth moderately while the other seven extracts only slightly inhibited fungal growth. There was some increase in antifungal activity after dialysis in extract 157F (crude 1.6, dialyzed 2.2).

In summary, except crude extract 157L (rating=4), none of the other crude extracts within this group exhibited strong to complete inhibition of

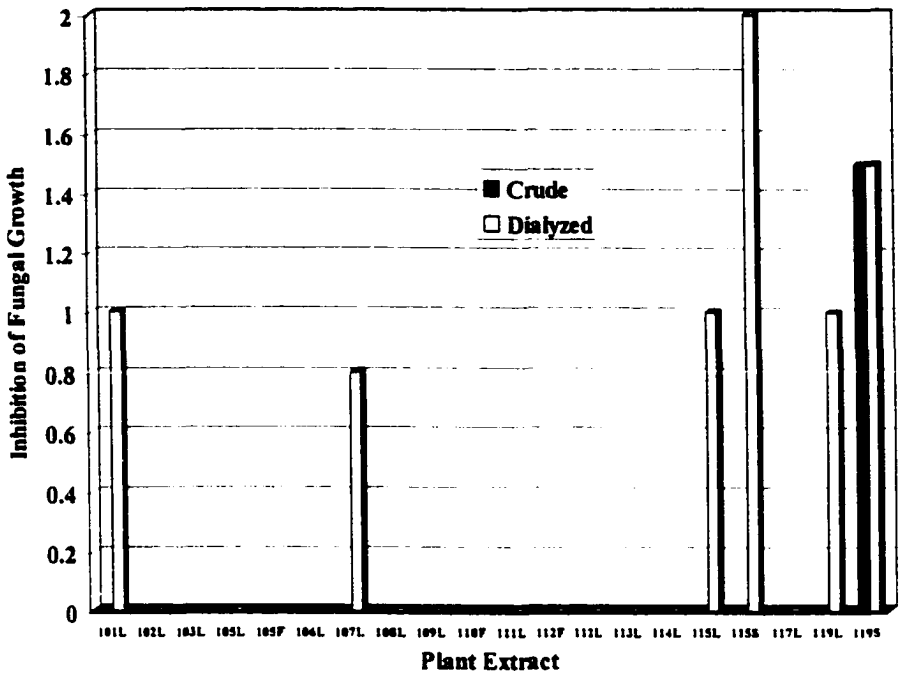


Figure III-8: Evaluation of Antifungal Activity of Crude and Dialyzed Extracts of PCN 101 to 119. Antifungal activity was measured using the standard assay with *F. chlamydosporum*. Rating of 4 = complete inhibition of conidial germination and hyphal growth, rating of 0 = no inhibition of fungal growth. (L = leaf, F = fruit, S = seed).

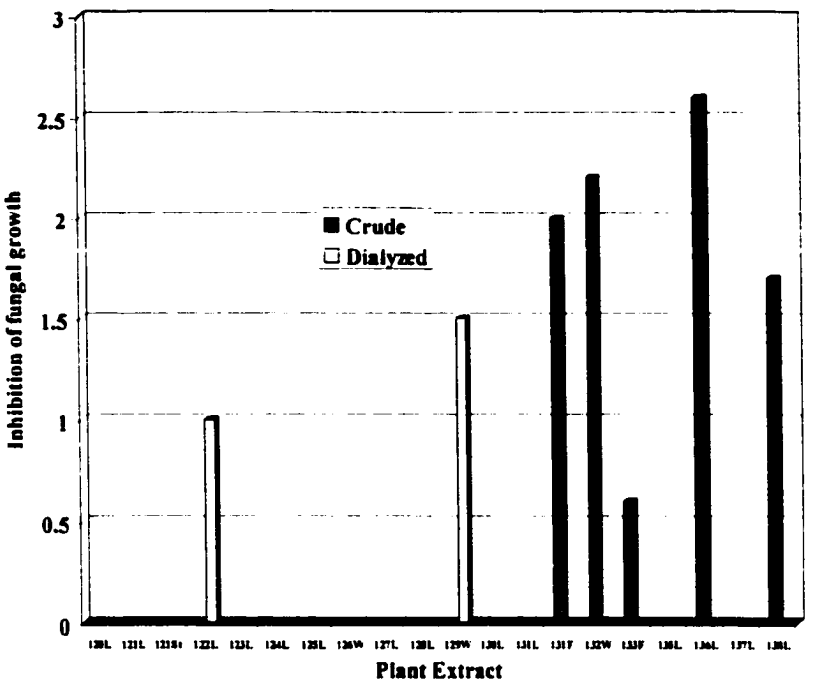


Figure III-9: Evaluation of Antifungal Activity of Crude and Dialyzed Extracts of PCN 120 -138. Antifungal activity was measured using the standard assay with *F. chlamydosporum*. Rating of 4 = complete inhibition of conidial germination and hyphal growth, rating of 0 = no inhibition of fungal growth. (L = leaf, St = stem, W = whole plant).

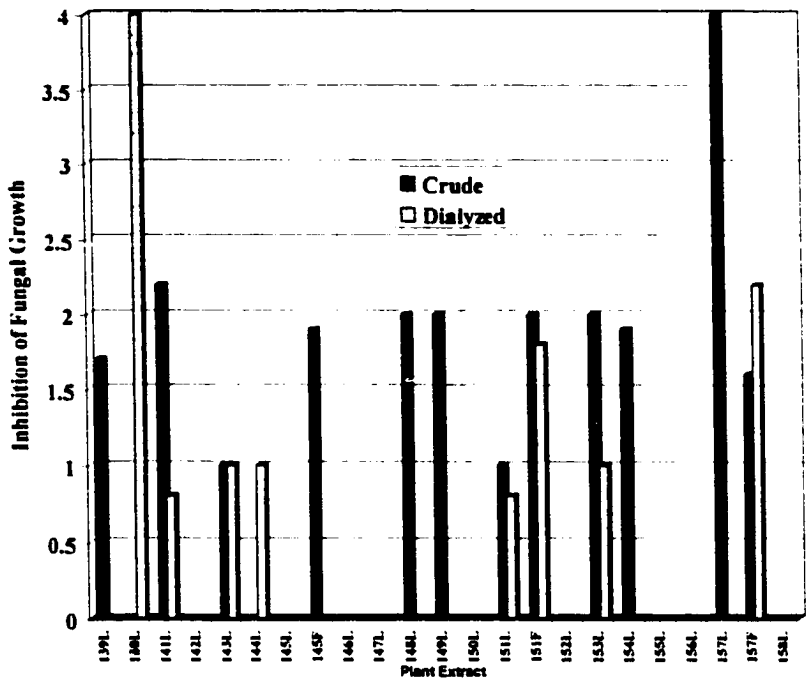


Figure III-10: Evaluation of Antifungal Activity of Crude and Dialyzed Extracts of PCN 140 -158. Antifungal activity was measured using the standard assay with *F. chlamydosporum*. Rating of 4 = complete inhibition of conidial germination and hyphal growth, rating of 0 = no inhibition of fungal growth. (L = leaf, F = fruit).

fungal growth. Unfortunately, 157L did not retain activity after dialysis. Surprisingly, while crude 140L did not exhibit antifungal activity, dialyzed extract completely inhibited fungal growth in the bioassay.

Time course study

A time course experiment was conducted to establish the optimum time for recording fungal growth in the bioassay. In this experiment, germination of conidia and hyphal growth were monitored over time using seven randomly selected dialyzed extracts (1S, 7L, 14L, 21F, 23L, 33L and 34LT) and a buffer control. Conidial germination was observed at 6, 18.5, 29, 40 and 48 h of incubation. There was no visible germination or growth after 6 h of incubation. Star shaped growth indicative of conidial germination was observed in treatments, containing extracts 7L, 14L, 23L, 21F or 33L after 18.5 h. After 29 h of incubation, the number of conidia that germinated increased dramatically in three of the extracts and the buffer control (Table III-1). In contrast, the number of conidia that germinated in wells containing 1S, 14L, 21F or 23L was much lower. After 40 h of incubation, moderate

Table III-1: Time Course of Conidial Germination and Hyphal Growth in the Bioassay.

Plant extract	Conidial germination		Hyphal growth rating	
	18.5 h	29 h	40 h	48h
1S	0	9	VL	3.8
7L	5	130	MG	2
14L	5	35	MG	2
21F	1	40	MG	2
23L	4	45	MG to HG	1
33L	5	130	MG	2
34LT	0	100	MG	2
Control	0	150	HG	0

Standard assay conditions were used in this experiment. Approximately 290 conidia were used in each well. Conidial germination and hyphal growth of *Fusarium chlamydosporum* was observed at 6, 18.5, 29, 40 and 48 h of incubation. The observation after 48 h was measured using the numerical rating scale outlined in the Materials and Methods. (MG = moderate hyphal growth, HG = heavy hyphal growth, VL = very little hyphal growth, S = seeds, L = leaves, LT = latex).

hyphal growth was noted in the buffer control and in all the extracts except 1S. In the case of extract 1S, conidial germination remained low (60 conidia) and there was only a small amount of hyphal growth. Although there was lower conidial germination in wells containing 14L, 21F or 23L after 29 h of incubation, the mycelial growth after 40 h of incubation was comparable to growth in the control. Hyphal growth covered most of each well and star shaped conidial germination was no longer visible after 40 hours of incubation in the control and all extracts except 1S. Even after 48 h, the number of germinated conidia remained the same as noted in the 40 h observation and the star-shaped growth pattern was still visible in wells containing 1S. Although there was some hyphal growth in this extract, the mycelia were branched, stunted and distorted.

Mycelial growth was extensive in the buffer control after 48 h of incubation and covered the total surface of each well of the microtiter plate. The buffer control received a rating of zero, indicating no inhibition of fungal growth. Mycelial growth remained moderate in extracts 7L, 14L, 21F, 33L and 34LT after 48 h and the growth was mostly in the center of the microtiter well. A fungal growth inhibition rating of 2 (moderate inhibition) was given to all these extracts. Hyphal growth was from moderate to heavy

in extract 23L indicating a slight inhibition of fungal growth. Therefore, a rating of 1 was given to extract 23L. No new conidial germination was observed in extract 1S and there was a clear indication that the hyphal growth had stopped. The hyphal growth was still distorted and the hyphae appeared to disintegrate into small fragments. For each extract, four replicates were used. Based on the observation, an inhibition of fungal growth rating of 3.5 to 4 was given to each replicate of dialyzed extract 1S. Average rating of four replicates was 3.8 indicating a very strong antifungal activity. Based on observations from the time course study, 48 h was selected as the standard incubation time for the fungal bioassay.

Range of activity

Several dialyzed extracts were tested for antifungal activity using *A. flavus* and *A. parasiticus*. The inhibition of growth of *A. flavus* and *A. parasiticus* by these extracts was similar in extent to the inhibition observed using *F. chlamydosporum*. For example, extract 34LT exhibited moderate and 8S exhibited slight inhibition of fungal growth with all three fungi.

DISCUSSION

One hundred-sixty-four crude extracts from over 100 tropical plant species were screened for antifungal activity against *F. chlamydosporum.* Thirty-five crude extracts exhibited anywhere from strong to complete inhibition of fungal growth. Twenty-five extracts showed very slight (rating of one or below) inhibition and in the rest of the extracts, inhibition of fungal growth was higher than one. After dialysis, most of the crude extracts lost their antifungal activity. Forty extracts retained antifungal activity after dialysis. Most of these extracts exhibited moderate to slight inhibition of fungal growth. Only five dialyzed extracts exhibited strong to very strong antifungal activity. Based on the results of this antifungal screen, we conclude that tropical rainforest species are a useful source of antifungal activities. The presence of putative antifungal proteins in the dialyzed extracts was less common.

Most of the extracts that exhibited antifungal activity before dialysis lost their activity after dialysis. Thus, it appears that most of the antifungal activity was due to soluble metabolites with molecular weights less than 3,500 Da. Apparently, macromolecular compounds with molecular weights

greater than 3,500 Da were responsible for the strong inhibition of fungal growth exhibited by the five dialyzed extracts.

Close observation of conidial germination and hyphal growth revealed that the mechanisms of inhibition fall into at least two classes. In the first class, conidial germination was completely inhibited after 48 h of incubation. In some cases, e. g. 98F, conidia did not germinate even after 72 h of incubation. In the second class, conidial germination did occur but the number of conidia that did germinate was often lower than in controls. In these cases, the reduction in germination was frequently coupled with a reduction or alteration in hyphal growth. For example, dialyzed extracts 1S and 115S strongly inhibited conidial germination and retarded hyphal growth, forming extremely branched and distorted hyphae.

In assays with 1S, none of the conidia germinated in the presence of crude extract. Only a few conidia germinated in assays containing dialyzed extract. In fact, hyphal growth stopped within two days and hyphal disintegration was observed. The inhibitory activity of these extracts may be due to enzymatic activity that interferes with hyphal growth. Mauch et al. (1988) reported that the inhibition of fungal growth in extracts of pea was caused by swelling and lysis of the hyphal tips of the fungus. When the same fungus was incubated with chitinase and β-1,3-glucanase purified from

pea, swelling and lysis of the fungal tips were observed. Fungal growth was also inhibited. Thus, extracts may exert their effect in at least three different ways. The first is to inhibit conidial germination, the second is to retard the growth of hyphae and the third is to distort hyphal growth.

The differences in the results obtained with crude and dialyzed extracts suggest that many of these plant extracts contain more than one antifungal activity. Indeed, many of these plants may possess a variety of defensive weapons that protect the plant from fungal attack. The observation that some extracts may utilize more than one mechanism of action, e. g. inhibition of germination and retardation of hyphal growth, support this conclusion. The observed differences in inhibitory activity between different plant extracts may reflect differences in the unit activity of the inhibitor and/or differences in the concentration of inhibitor in the extract.

The inhibitory activity may not be stable over time. The inhibitor may break down naturally. Alternatively, it may be inactivated or detoxified through the action of 1) endogenous activities in the extract, i. e. hydrolases; 2) reactive components in the extract, i. e. phenolic compounds, oxygen; or 3) activities present in the fungus, e. g. hydrolases, detoxification mechanisms. This may account for the reduced activity or loss of activity

with time noted in some extracts. The extraction conditions used in these studies were not designed to protect sensitive or unstable activities. Antioxidants, metal chelators, protease inhibitors and/or reductants were not included in the buffer. The only protectant used was PVP that was added to reduce the concentration of potentially reactive phenolics.

The activities noted in these screens may not reflect all of the activities initially present in the extracts. Protein precipitation and denaturation continue during storage and dialysis of the extracts. The loss of activity upon dialysis is consistent with the conclusion that metabolites were responsible for some fungal growth inhibition. In a few instances, the activity actually increased after dialysis. The inhibition or masking of activity by endogenous factors that are removed upon dialysis may explain this observation. Certainly, only a relatively small proportion of plant species tested exhibited strong antifungal activity.

The activities detected in these aqueous extracts do not reflect the total antifungal activity present in the plant tissue or the potential to produce defense-related proteins and metabolites in response to fungal invasion. Many antifungal metabolites may not be soluble or may be only sparingly soluble in aqueous extracts. Normally, organic solvent extraction is required to isolate those compounds. Frequently, plant defenses are not expressed

constitutively but are produced in response to attack. We have not attempted to induce any of the plant species tested to enhance the occurrence or levels of defensive compounds present in the tissue.

In many plants, defense-related proteins are induced upon infection by pathogens (Benito et al., 1998; Bol et al., 1990; Dubery and Slater, 1997; Hu et al., 1997; Hu and Reddy, 1997; Kragh et al., 1995; Mohr et al., 1998; Rasmussen et al., 1992; Rethinasamy et al., 1998; Yi et al., 1996). These defense-related proteins include enzymes responsible for the production of phytoalexins and other defensive metabolites, as well as pathogenesis-related proteins such as chitinase, glucanase and protease inhibitors. It is possible that extracts without antifungal activity are from plants that do not produce defense-related molecules constitutively and have not been induced. Therefore, it can not be concluded from this antifungal screening that the extracts that did not exhibit any antifungal activity are from plants that don't produce any antifungal compounds. Even though antifungal compounds are not produced constitutively by these plants, they may very well possess the ability to activate defense genes in response to various elicitors. There may be an ecological advantage for plants that do not produce defense-related compounds constitutively. Plants with inducible defenses would dedicate

resources to the production of defensive agents only when required, conserving resources for growth and reproduction.

Selected extracts were screened against three different fungi. All of these selected extracts exhibited similar antifungal inhibition against all three fungi. From this study, it can not be concluded that similar results would be obtained if the rest of the extracts were tested with the same fungi or other fungi. Antifungal compounds present in the extracts may or may not have a broad range of activity. The activity may vary for different fungi due to different modes of action or different fungi may be more or less sensitive to certain defense compounds or may be able to detoxify or counteract certain types of defenses.

All the extracts were tested against *F. chlamydosporum*, which belongs to the higher group of fungi. None of these extracts was tested against any representatives from the lower group of fungi such as *Phytophthora* sp. or *Pythium* sp. There are many antifungal proteins such as PR-1 (see review) and PR-5 (Woloshuk et al., 1991) proteins that specifically affect the lower group of fungi such as *Phytophthora infestans*. Consequently, if these 164 extracts were tested for antifungal activity against members of the lower group of fungi, the results may be completely different from the results obtained in this antifungal screen. The goal of this

section was to identify tropical plants with antifungal activity. For this reason, a large number of plant extracts was screened. To facilitate screening and the interpretation of results, it was convenient to use only one species of fungus in the bioassay.

CONCLUSIONS

Our objective was to discover and purify antifungal proteins/polypeptides from tropical plant extracts. In this initial screen, we identified a number of tropical plant extracts with antifungal activity. The antifungal activity in several plant extracts was retained after dialysis. Presumably, antifungal activities retained after dialysis might be due to proteins or polypeptides. Based on these results, efforts were initiated to further purify these putative antifungal proteins from the plants identified.

Chapter IV: Preliminary Characterization and Properties of Antifungal Activities from Seeds of *Swartzia simplex*

INTRODUCTION

During the initial screen, a number of extracts were identified that exhibited moderate inhibition of fungal growth. One of these was PCN 119, a small tree species from the lowland tropical rainforest of Costa Rica. This plant was identified as *Swartzia simplex* (Swartz). *S. simplex* is a member of the Caesalpinioideae, one of three subfamilies of the Leguminosae. Activity in seed extract of *S. simplex* was retained after dialysis indicating that the active components were greater than 3,500 Da in size.

Seeds of *S. simplex* was collected by Dr. Karel R. Schubert from the La Selva Biological Station in the Limon Province of Costa Rica. The field station is operated by the Organization for Topical Studies. According to Dr. Schubert's field observations, fruits of *S. simplex* remain on the tree for approximately 4-6 months. During this period, there were no visible signs of fungal infection or insect predation. Subsequently, an extract of *S. simplex* seed was tested against *Aspergillus flavus* and *Fusarium*

moniliforme. This extract was not included among the selected extracts tested against *A. flavus* and *A. parasiticus* in the initial screen (Chapter III). The antifungal activities of the selected extracts tested in the initial screen against *A. flavus* and *A. parasiticus* were similar to those obtained with *F. chlamydosporum*. As discussed in Chapter III although the results of the selected extracts were similar with the three fungi tested, results with different extracts and/or different fungi might be quite different. This was the case with *S. simplex* seeds. The latter exhibited only moderate inhibition of *F. chlamydosporum* growth but strongly inhibited growth of *A. flavus* and *F. moniliforme*. The activity was retained after dialysis and inhibition of both by dialyzed extract was strong. Based on these observations the decision was made to further purify the antifungal activity from *S. simplex* seeds.

The purification of bioactive factors was initially based on results from bioassays using three different fungi, *A. flavus, F. moniliforme* and *F. chlamydosporum*. In the early studies, it was clear that after initial purification the growth of *F. chlamydosporum* was extremely sensitive to a variety of factors present in these extracts. In contrast, the growth of *A. flavus* and *F. moniliforme* were not affected by many of the components

(fractions) which retarded the growth of *F. chlamydosporum*. In addition, *F. chlamydosporum* is a saprophytic/opportunistic pathogen and our goal was to purify antifungal activities against agronomically important fungal pathogens.

From the beginning, it was clear that there were multiple components with antifungal activity, in extracts of seeds of *S. simplex* and the activities were different against different fungi. It was not possible to pursue the purification of all the different activities against both *F. moniliforme* and *A. flavus*. Thus, we had to concentrate on purifying the activities against one fungus. *A. flavus* is one of the most difficult fungi to control and this fungus produces aflatoxin which is very toxic to animals and humans. Therefore, we decided to concentrate our efforts on the purification of antifungal proteins active against *A. flavus*. The later stages of purification were based entirely on the results of the *A. flavus* bioassays.

MATERIALS AND METHODS

Fungal bioassay

The antifungal assay was performed using the liquid bioassay according to the procedure described in Chapter III. The pathogenic fungus, *A. flavus*, was used for all bioassays, unless noted.

Extraction of plant tissue

Neutral extraction: Plant seeds were routinely extracted in 10 mM Tris-HCl, pH 8.0 according to the extraction protocols described in Chapters III. After homogenization, filtration and centrifugation, the crude extract was dialyzed using Spectra Por 3 dialysis membrane with a nominal molecular weight cut off of 3,500 Da (3500 MWCO). The dialyzed supernatant was referred to as the neutral extract (NEx).

Acid extraction: Seeds of *S. simplex* were homogenized in 5 volumes of 0.1 N HCl. Typically, the homogenized tissue was filtered and centrifuged as per the neutral extraction protocol. After centrifugation, the acid extract was either 1) dialyzed exhaustively against 10 mM Tris-HCl (pH 8.0) using a 3,500 MWCO membrane; or 2) neutralized with the

dropwise addition of 2 M NaOH; or 3) neutralized and then dialyzed. For 2 and 3, the extract was held on ice for one h after neutralization. After one h, the cloudy supernatant was centrifuged (20,000 x g for 10 min) to remove the denatured protein and the clarified supernatant was filtered through Whatman 3MM filter paper. The dialyzed acid extract (not neutralized) was referred to as acid extract (AEx). The neutralized acid extract (not dialyzed) and the neutralized/dialyzed acid extract was referred to as neutralized acid extract (NAEx) and neutralized/dialyzed acid extract (NDAEx), respectively. All extracts were stored at 4 °C or frozen at –20 °C.

Heat treatment

To determine the stability of the active components to heat denaturation, neutral extract was subjected to heating at 60, 70, 80, 90, and 100°C for 3 min. After heat treatment, the extracts were cooled quickly on ice. To remove precipitated protein, the heat-treated samples were centrifuged at 18,000 rpm for 20 min. The pellet was discarded and the soluble material present in the supernatant was assayed for antifungal activity.

Preparative isoelectric focusing

A BioRad Rotofor preparative isoelectric focusing cell was used for preparative isoelectric focusing. Crude dialyzed extract was dialyzed against 10 mM sodium chloride prior to isoelectric focusing. Twenty-six ml of the dialyzed extract was added to 12.5 ml of 25% glycerol. To this solution, 0.62 ml of ampholyte, pH range 3-10 (BioRad), was added bringing the total volume of the solution to 39.1 ml. The final volume of the solution was brought to 50 ml by adding 10.9 ml of deionized water. The entire sample containing dialyzed crude extract, glycerol, ampholyte and deionized water was injected into the Rotofor cell. Isoelectric focusing was performed according to the BioRad IEF protocol provided by the manufacturer.

Twenty fractions (2.5 ml each) were collected and the pH of each fraction was measured with a Beckman micro-electrode. All 20 fractions were dialyzed against 1 M NaCl to disrupt the electrostatic forces between ampholytes and proteins. After dialysis with high salt, fractions were exhaustively dialyzed against 10 mM Tris-HCl (pH 8.0) and assayed for antifungal activity.

Size-exclusion chromatography (SEC)

A 100-cm x 1.6-cm Pharmacia column was packed with Pharmacia prep-grade Superose 12 or Sephacryl-200 HR gel filtration media according to the manufacturer's instructions. Once packed, each column was equilibrated at a flow rate of 1 ml/min with 10 mM Tris-HCl, pH 8.0, using a Pharmacia FPLC system equipped with a UV monitor and conductivity flow cell. Generally, the sample was concentrated (2-4 fold) by ultrafiltration (Centricon 10,000 MWCO) before loading onto the column. The concentrated sample was centrifuged in a microfuge just prior to injection to remove any insoluble material and 0.5 ml of the sample was applied to the column. Proteins were eluted with the same buffer and collected in 4-ml fractions. The absorbance of eluted fractions was measured at 280 nm.

Chitin-affinity chromatography

Neutral chitin column: Sigma practical grade crab shell chitin (Sigma # C-9213) was poured into a 1-cm x 15-cm column and washed extensively with each of the following: 0.1 M HCl, deionized water, 0.1 M NaOH and 1 M NaCl. After washing, the column was equilibrated with 10 mM Tris-HCl, pH 8.0. Neutral extract (5-20 ml) was loaded onto the column and the unbound fraction, i.e. flow through (FT), was collected and reapplied to the column four times. The final flow through was collected and stored at 4 °C. The column was washed sequentially with 10 mM Tris-HCl, pH 8.0 (1st Tris wash), 0.1 M NaCl in 10 mM Tris-HCl (0.1 M salt wash) and 1 M NaCl in 10 mM Tris-HCl (1 M salt wash).

Proteins that were bound to chitin were eluted with 0.1 M acetic acid. The absorbance of each 10-ml fraction was measured at 280 nm with a Beckman 7500 Diode Array Spectrophotometer and the peak protein-containing fractions were pooled. Protein in the pooled fractions was concentrated by ammonium sulfate precipitation (i.e. 0.6 g of powdered enzyme-grade ammonium sulfate was slowly added per ml of sample with gentle stirring. The sample was held on ice for 30 min and then centrifuged

at 18,000 rpm for 20 min). The pellet obtained after ammonium sulfate precipitation was resuspended in a minimal volume of 10 mM Tris-HCl, pH 8.0, and the supernatant fluid was saved.

After acetic acid elution, the column was washed with 100 ml of 10 mM Tris-HCl, pH 8.0 (2nd Tris wash). Protein bound to the column but not removed with 0.1 M acetic acid was eluted with approximately 100 ml of 0.1 M NaOH. Peak fractions were pooled and concentrated in the same manner as described for the acetic acid eluate. The salt washes, Tris washes, supernatant fluids after ammonium sulfate precipitation and the resuspended pellets (eluates) were dialyzed against 10 mM Tris-HCl (pH 8.0).

In some instances, a modification of the neutral chitin column chromatography protocol was used. After the first Tris wash, bound proteins were eluted stepwise with 0.1 M, 0.2 M, 0.3 M, 0.4 M, 0.5 M and 1 M NaCl in 10 mM Tris-HCl, pH 8.0. After elution, fractions were pooled, concentrated by ammonium sulfate precipitation and dialyzed in Tris-HCl buffer.

Acid chitin column: Chitin was packed into a column and washed extensively as described above for the neutral chitin column. After washing, the column was either equilibrated with 20 mM sodium acetate buffer, pH 5.6 or 50 mM sodium acetate, pH 3.8 for dialyzed acid extract (AEx) and acid extract (NAEx), respectively. Five ml of the extract was loaded onto the column and the flow through was collected and reapplied to the column four times. The final flow through was collected and saved.

The column was washed sequentially with column equilibration buffer, 0.1 M NaCl and 1 M NaCl in buffer. Bound proteins were eluted first with 0.1 M acetic acid and then with 0.1 M NaOH. Between the low and high pH elution, the column was washed with either 1 M salt in buffer (neutral chitin column) or equilibration buffer without salt in the case of the chitin column where dialyzed acid extract was used. Eluates were precipitated with ammonium sulfate as described for the neutral chitin column. The ammonium sulfate precipitated pellets obtained after elution with 0.1 M acetic acid elution (acetic acid eluate) and 0.1 M NaOH elution (NaOH eluate) were resuspended in a minimal volume of 10 mM Tris-HCl, pH 8.0. The washes, supernatant fluids and pellets were dialyzed against 10 mM Tris-HCl, pH 8.0 to remove excess NaCl and ammonium sulfate.

Samples were concentrated by ultrafiltration using Centricon 10 concentrators as noted.

Ion-exchange chromatography

Anion-exchange chromatography was performed using a Pharmacia Resource Q (RSQ) column. Before injection of the sample, the column was washed with 1 M NaCl in 10 mM Tris-HCl, pH 8.0 at a flow rate of 1 ml/min and equilibrated with 10 mM Tris-HCl, pH 8.0. Before loading onto the RSQ column, the sample was dialyzed against 10 mM Tris-HCl, pH 8.0. After sample injection, the column was washed with several volumes of column buffer to remove unbound proteins. Bound proteins were eluted with a programmed gradient from 0 to 1 M NaCl in 10 mM Tris-HCl, pH 8.0, at a flow rate of 1 ml per min. Absorbance of the eluted proteins was measured at 280 nm. All RSQ column fractions were dialyzed exhaustively against 10 mM Tris-HCl (pH 8.0) to remove salt.

Determination of purity

Protein purity was assessed by SDS-PAGE (12.5% total acrylamide) according to Laemmli (Laemmli, 1970). Protein samples were denatured by boiling for five min in SDS sample buffer containing β-mercaptoethanol

(BME). After electrophoresis, gels were stained with Coomassie Brilliant Blue R 250 and then destained for 48 h in destaining solution. After destaining, the gels were restained with silver nitrate according to the method described by Bloom, 1987.

Hemagglutination

Lectins were detected by measuring hemagglutination. The agglutination assay was performed according to the protocol described by Lis and Sharon (Lis and Sharon, 1981). Three different sources of red blood cells were used for the agglutination assay: 1) human type A red blood cells (RBC); 2) porcine RBC; and 3) rabbit RBC. For each fraction, 100 µl of concentrated sample (Centricon 10) was serially diluted with phosphate buffered saline (PBS) in a 96-well, U-bottom microtiter plate. Red blood cells were added to these diluted samples and the plates were incubated for 30-60 min. After incubation, agglutination of red blood cells was measured visually and the data was recorded. Positive agglutination resulted in a dispersed appearance to the red blood cells whereas a negative result produced a compact dot of RBC.

Enzyme assays

Spectrophotometric chitinase assay: Endochitinase activity was measured spectrophotometrically using a modification of the method developed by Boller and Mauch (Boller and Mauch, 1988). Individual fractions (100 µl) were incubated in a water bath with 0.01% (w/v) insoluble shrimp shell chitin (Sigma # C8908) resuspended in 10 mM NaAc, pH 5.0, for 1 h at 37°C. After incubation, the insoluble chitin was removed by centrifugation in a microfuge at 14,000 rpm for 5 min. A portion of the supernatant fluid (175 µl) was pipetted into glass tubes containing 10 µl of N-acetyl glucosaminidase (Sigma # A 3189) and incubated in a 37°C water bath for another 2 h. After incubation, 35 µl of 1 M borate was added to the reaction mixture and the reaction mixture was heated in a boiling-water bath for 3 min and then cooled to 25°C. One ml of color reagent (p-dimethylaminobenzaldehyde; DMAB) was added to the cooled reaction mixture and the latter was incubated at 37°C for 20 min. After the final incubation, the absorbance of the reaction mixture was measured at 595 nm. N-acetyl glucosamine (Sigma # A 8625) was used as a standard and 10 µl of a 0.5 mg/ml solution of chitinase (Sigma # C 7809) in 10 mM NaAc (pH 5.0) was used as a positive control.

β-1,3-Glucanase Assay: A modification of the spectrophotometric assay developed by Abeles and Forrence (Abeles and Forrence, 1970) was used to measure β-1,3-glucanase activity. A sample (100 µl) from each fraction was incubated at 37°C for 3 h with 100 µl of 2.5% (w/v) laminarin (Sigma # L-9634) in 125 mM sodium acetate buffer, pH 5.0. After incubation, 750 µl of color reagent (dinitrosalicylic acid) was added to the reaction mixture and the latter was heated for 5 min in a boiling-water bath. The reaction mixture was cooled to room temperature, diluted (1:10) with deionized water and the absorbance of the samples was measured at 500 nm. A standard curve was constructed using 10 mM glucose and 100 µl of 5 mg/ml laminarinase (Sigma # L 5144) was used as a positive control.

Chitinase and glucanase in-gel assay

To detect different isoforms of chitinases and glucanases, polyacrylamide gel electrophoresis was performed under nondenaturing conditions. Native PAGE without SDS was carried out at high pH (pH 8.9) according to Davis (Davis, 1964). The acrylamide concentration used for all gels was 12.5% unless otherwise noted. Ten to 15 µl of each sample was added to an equal

volume of sample buffer, before loading onto the gel. The gel was subjected to electrophoresis at 20 mA for 3-4 h at room temperature. After separation of protein bands on the native gel, the presence of chitinase and glucanase bands on the resolving gel were detected using the procedure developed by Shen and associates (Shen et al., 1991). After electrophoresis, the resolving gel was incubated at room temperature for 5 min in 0.1 M NaAc buffer, pH 5.0.

After equilibration in acetate buffer, the gel was overlaid with a precast 7.5% polyacrylamide gel containing 0.01% (w/v) glycol chitin. The gels were wrapped in saran wrap and incubated for 45-60 min at 37°C. After incubation, the original native gel was separated from the overlay gel. The later was incubated at room temperature for 5 min in a freshly prepared solution of 0.01% (w/v) fluorescent brightener 28 (Sigma # F 3543) in 0.5 M Tris-HCl-Cl, pH 8.0. The gel was then transferred to deionized water for 5 min at room temperature to remove excess brightener and the gel was viewed with an UV transilluminator. Chitinase bands appeared as non-stained (darker) spots.

To detect β-1,3-glucanase activity, the 12.5% native polyacrylamide gel from above was washed in deionized water for 5 min and then incubated in 0.1 M NaAc (pH 5.0) for 5 min. After equilibration with NaAc, the gel

was incubated at 40°C for 2-3 h with a mixture containing 75 ml of 0.05 M NaAc (pH 5.0) and 1 g of laminarin dissolved in 75 ml water. At the end of this period, the gel was transferred to a solution of methanol:acetic acid:water (5:5:2). Subsequently, the gel was washed with water and stained with freshly prepared 2, 3, 5-triphenol tetrazolium chloride in 1 M NaOH by heating in a microwave for 3 min with shaking every 30 seconds.

RESULTS AND DISCUSSION OF PRELIMINARY PURIFICATION

Approach to purification of antifungal proteins and other factors

Before discussing an approach to purification of bioactive materials, one needs to establish what the goals of purification are and how purification is to be defined. The goal of purification is to obtain a specific material in relatively pure form from a heterogeneous mixture of similar and dissimilar materials. The purification process itself is based on the selective removal of a specific substance from the mixture or the reverse, i. e. the removal of contaminants from the mixture. Purification can be achieved using either positive or negative selections. The ultimate goal is to obtain enough material in a state of purity suitable for the elucidation of the physical and chemical structure of the bioactive agent.

There are two basic approaches to purification of bioactive materials: 1) the bioassay-directed approach and 2) the biochemistry-based approach. The purification of antifungal activity by necessity focuses on the biological activity of these factors, i. e. their ability to inhibit or retard the growth, development and replication of a specific fungus. Thus, much of the purification described herein has been based on the bioassay-directed

approach. Various factors contribute to the complexity of this approach. These factors include but are not limited to the following: 1) biological variability in fungal growth responses; 2) time required for the bioassay; 3) qualitative nature of the bioassay; and 4) inability to distinguish between differences in the specific type of activity (e. g. occurrence of multiple forms or types of activities that give the same response, i. e. the "inhibition of fungal growth"). In addition, not all inhibitors are generic. Many are very specific, i. e. a component may inhibit one fungus but not another. For example, certain extracts tested in Chapter III inhibited the growth of *F. chlamydosporum* but not *F. moniliforme.*

The ability to identify specific biochemical attributes associated with a biological activity ("handles") can reduce the complexity to some extent. For example, the purification of chitinase may be more direct because there are biochemical tests that allow one to specifically measure chitinase activity in the presence of a mixture of other antifungal proteins. In this case, chitinase may or may not exhibit antifungal activity in the specific bioassay being used, for instance activity against *A. flavus*. These tests are also capable of distinguishing specific isoforms of chitinase without the requirement for them to have a direct effect on the fungus. With the biochemistry-based approach, the higher degree of specificity may be

actually be a drawback. The concern is that other bioactive factors which do not have a measurable and known biochemical activity will be missed by this more limited examination.

To be able to purify anything one must have 1) a way to measure the substance or activity (i. e. concentration) and 2) a way to assess the state of purity. In the case of proteins, the latter is most often done using SDS PAGE or other techniques such as HPLC, mass spectrometry, amino acid sequencing and isoelectric focusing. One can measure either the presence of the desired substance or the absence of contaminating substances or both. The former, however, is often not so simple when isolating bioactive molecules.

By definition, bioactive factors are substances that have some measurable biological or biochemical activity. As stated earlier, purification can be based on the isolation of these biological activities (bioassay-directed approach) or on a biochemical assay (biochemistry-based approach). Purification is somewhat simpler if there is a biochemical measure of activity, e. g. an enzyme assay, immunoassay, agglutination assay or enzyme inhibition assay. Life is not so simple when the biological activity is a physiological or cellular response, e. g. measuring effectors of cell growth, cell division or cell development.

Measurements may be quantitative (e. g. enzyme assay, enzyme inhibitor assay), semi-quantitative (e. g. agglutination) or qualitative (e. g. inhibition of fungal growth assay). The fungal bioassay used throughout this study is a fungal growth inhibition assay. Fungi can be grown on solid media in the presence of crude extracts or purified components. These materials can be applied to a filter disk placed on the media (disk assay) or introduced into a well cut out of the media. Activity is measured as a zone of clearing, i. e. inhibition of growth of hyphal mat on the surface of the media. Normally, the width of the zone of inhibition provides a semi-quantitative measure of activity. Unfortunately, this technique which has been used effectively to identify antibiotic natural products does not perform as effectively when the bioactive material is a high molecular weight protein. Proteins do not diffuse into the media and, as a consequence, give a very narrow zone of inhibition.

For this reason, we have exclusively used a liquid growth media to which potential inhibitory proteins can be added. The assay can be easily replicated. The only limitation is the amount of material available for assay and the time involved in setting up and recording the results of the assay. The latter is quite tedious and has to be done by direct observation using an inverted microscope. Because all of the plant materials tested in these

studies are limited and, therefore, individual fractions of more purified components are even more precious. The major limitation is that the assay is only qualitative, not quantitative. Greater reliability can be obtained by replication and by determining the dose response for individual fractions. Unfortunately, this requires much more material. Although the use of replicated treatments with different concentrations provides a semi-quantitative analysis, the assessment of growth is still only qualitative.

Attempts have been made to make the microtiter plate assay more quantitative by measuring fungal "growth" spectrophotometrically in a microtiter plate reader. Still, many factors interfere with the reliability and accuracy of these methods. Ultimately, a plate reader was not available for these studies and we were forced to rely on the qualitative assay. Additional quantitative information can be derived by actually measuring and recording the number of conidia that germinate. This process is extremely tedious and time consuming. General observations on conidial germination and the morphology of fungal growth (stunting, hyperbranching, etc.) however, are extremely useful.

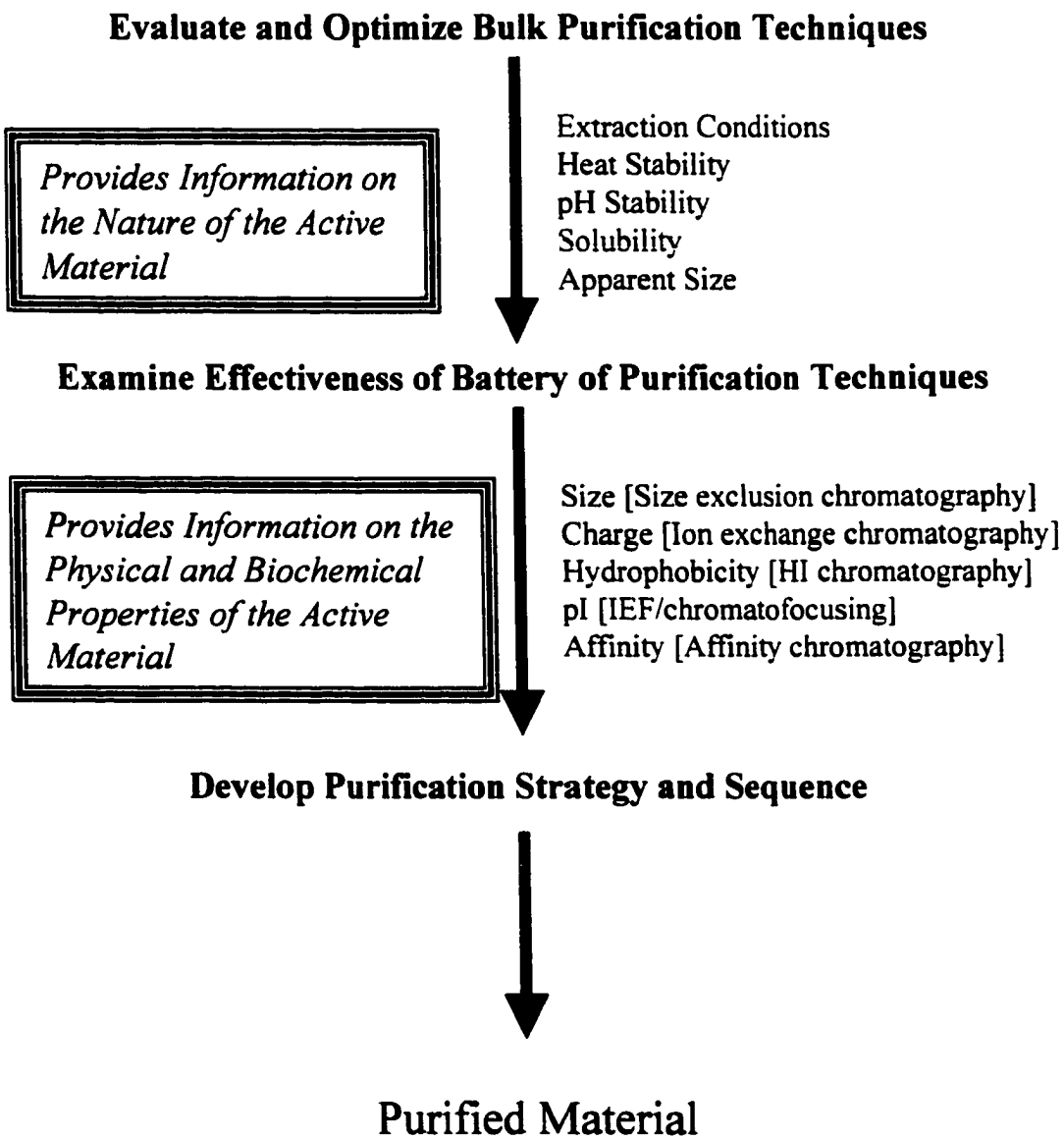
Because of the qualitative nature of the assay, one does not know whether differences in activity reflect differences in the amount of material present in an extract or on the unit activity of the material or both. Thus,

when confronted with two samples with equivalent or different activities, it is difficult to choose to pursue one activity or the other. One may be present at very low concentrations but have a very high unit activity or just the opposite. In either case, the combination of concentration and unit activity can give the full range of inhibition ratings. Indeed, it may be better to pursue a fraction with a fungal growth inhibition rating of 2.5 over a fraction with a rating of 4. There are other complications. In some cases, a threshold amount of material may be necessary to detect activity. In other cases, activity may be in low abundance and masked by other components in the mixture.

The general approach to protein purification used in these studies involves three basic steps 1) the evaluation and optimization of bulk methods; 2) the preliminary evaluation of purification techniques; and 3) the development of a purification strategy or scheme. These steps are outlined in Figure IV-1 and the rationale behind each step is discussed below.

Research in Dr. Schubert's laboratory is focused on the identification of novel bioactive proteins and natural products. Extraction conditions are used that favor the isolation of proteins, especially durable proteins that are

Figure IV-1: Approach to the Purification of Bioactive Proteins



capable of surviving in harsh environments such as the surface of a leaf, the seed coat or the gut of an insect. To select "tough" proteins, plant tissues are extracted with low ionic strength, neutral pH buffers with minimal additives. The only "protectant" added during extraction was PVP. The latter is added to remove phenolic compounds that react with proteins contributing to protein denaturation and causing chemical modifications. These modifications complicate interpretation of results by introducing molecular heterogeneity. This heterogeneity may create apparent "isoforms" or cause spurious results with techniques such as amino acid sequencing, isoelectric point determination and molecular weight determination.

This is the standard extraction protocol. In some instances, there are advantages to the use of different or more "stringent" extraction procedures, e. g. extraction at low or high pH. Extraction with low (deionized water) or high ionic strength buffers or with organic solvents, additives and extractants such as chaotropic agents (urea, guanidine) or ionic or nonionic detergents are also possible. Potential advantages of these modifications are that a different range or complement of bioactive factors may be isolated using different extraction protocols. At the same time, certain contaminants may not be extracted. Another potential benefit may be that some contaminants

may not be stable using the modified extraction protocol and can be removed from the more stable bioactive materials.

Once conditions are identified for the extraction of a bioactive factor, attempts can be made to optimize extraction conditions and thereby enhance extraction efficiency (total activity), recovery (yield) and/or purity (specific activity) of the starting material. This is especially useful with certain enzymatic activities that tend to be more sensitive to external conditions. Because the focus of this work is on the discovery of relatively robust activities, little effort has been spent on optimizing extraction conditions The use of drastically different extraction conditions, e. g.. Tris-HCl buffer, pH 8 vs. 0.1 M HCl, have been used on the premise that a different complement of activities may be isolated or less stable contaminants may be removed. This approach also provides information that may help identify specific active components.

The next step is to examine the effectiveness of bulk purification techniques that may be useful for the first steps of the purification. Bulk purification techniques are procedures used for the initial fractionation of the dilute crude extract. The goals of bulk purification are: 1) remove as much of the contaminating protein as possible from the relatively dilute extract without substantial loss of the desired activity; 2) concentrate the desired

activity into a more manageable volume; and 3) provide basic information and insight on the general properties of the active material that may be useful later in the purification.

The primary bulk technique used in these studies was dialysis. This technique can provide information concerning the nature of the active material, e. g. is it a low molecular weight compound or metabolite or is it a higher molecular weight component like a protein. In addition, dialysis is a relatively effective way to remove interfering substances like phenolic compounds with molecular weights below a certain cut-off. Other bulk techniques are listed below in Table IV-1. Heat treatment and ultrafiltration were routinely evaluated for their potential use as bulk purification techniques.

Table IV-1: Examples of Bulk Purification Techniques.

	Purification	Concentration	Information
Technique			
Heat Treatment	Yes	No	Yes
Ultrafiltration	Yes	Yes	Yes
Dialysis	Yes	No	Yes
Stability (pH)	Yes	(Yes)	Yes
Solubility[1]	Yes	Yes	Yes

[1] Solubility precipitation with ammonium sulfate, PEG, ethanol, acetone.

Once bulk purification techniques have been evaluated and exploited, if possible, the next step is to evaluate generic techniques that separate molecules based on general properties of biomolecules. These properties and the associated separation techniques are listed below.

1. Size	Size-exclusion (gel filtration) chromatography
2. Charge	Anion and cation-exchange chromatography
3. Hydrophobicity	Hydrophobic interaction chromatography
4. Isoelectric point	IEF/ chromatofocusing
5. Affinity	Affinity chromatography

Again, these techniques were screened for effectiveness as preliminary purification techniques. The techniques were evaluated based on the following criteria:

1. Yield
2. Fold purification
3. Purity of final material
4. Ability to resolve differences in activities and/or isoforms of the same activity.

Certain techniques such as gel filtration may introduce secondary separation techniques. For example, gel filtration media also introduce potential ionic, hydrophobic and affinity interactions that may play a role in specific separations. Each technique can be optimized by selection of specific chromatography media and separation conditions (pH, buffer, buffer concentration, additives such as salt, ionic strength, etc.).

Each technique also provides information about the biochemical and structural properties of the active material. This information along with an analysis of the performance of the technique and the nature of contaminating substances is used to develop a purification protocol including the sequence of steps, combining one or more techniques to achieve further purification. In this process, two factors need to be kept in mind. The first is that the purification sequence is important and alterations in sequence can alter the final purification. Second, with the sensitivity of modern analytical techniques such as HPLC, mass spectrometry, automated protein sequencing, one does not have to purify a protein to homogeneity in order to characterize the protein. One only needs to purify the material enough to be able to identify the putative bioactive protein. Furthermore, some methods, for instance the chitinase in-gel assay are not sensitive to contaminating

activities such as hemagglutination, while others may be extremely sensitive to contaminants, e. g. protein sequencing. Note: combining techniques such as SDS-PAGE with electroblotting onto PVDF membranes can circumvent this limitation in the case of protein sequencing.

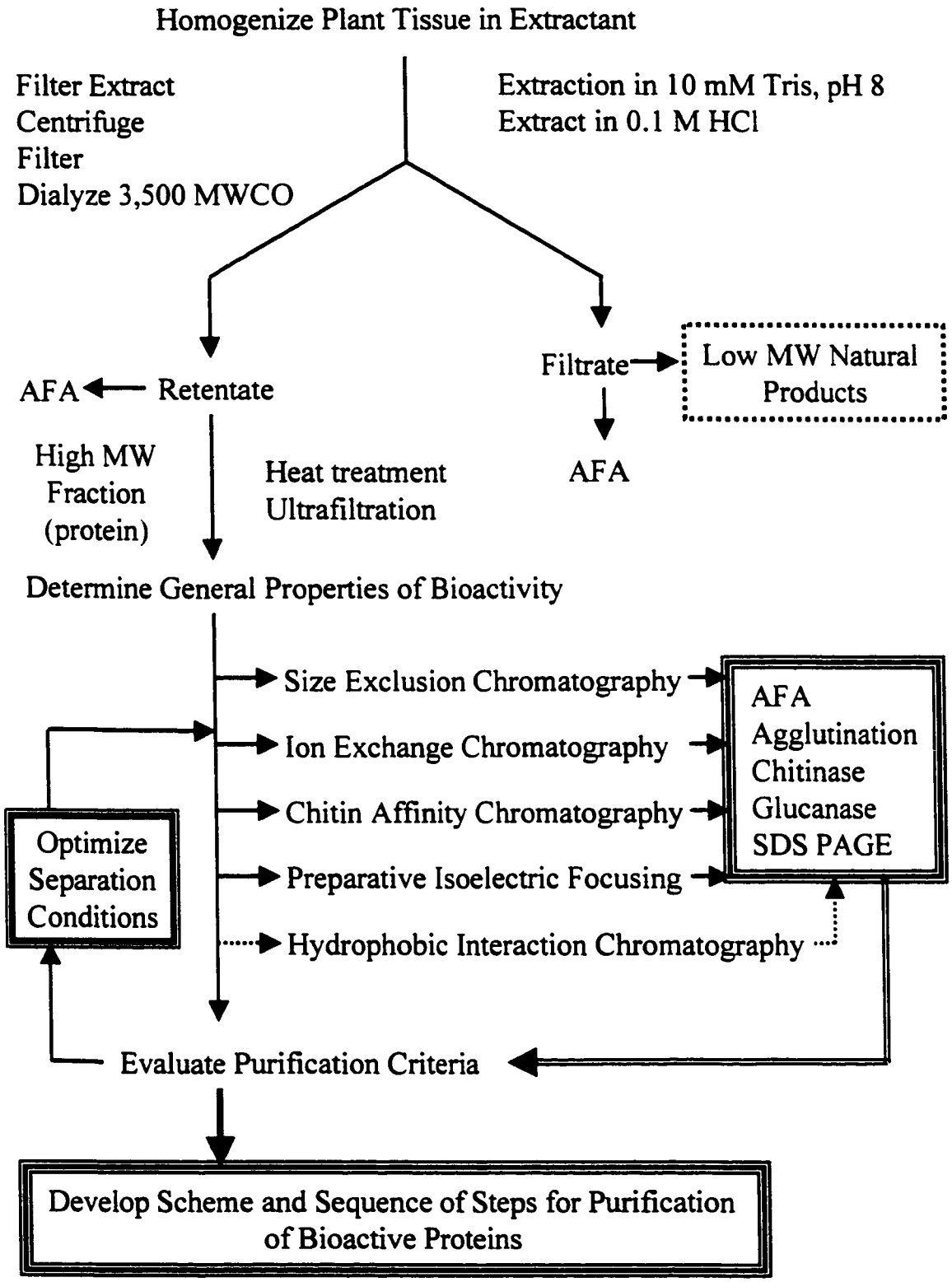
Potential pitfalls of these techniques are 1) activities may associate and co-purify with one another; 2) active components may require more that one factor for activity or multiple activities may act in a synergistic manner, e. g. chitinase and glucanase; and 3) one may measure the properties of a minor contaminant that has for example an abundance of certain amino acid residues. Alternatively, because of the sensitivity of some assays including certain bioassays, a minor component, not the major component, may actually be responsible for biological activity.

Other factors to consider include the availability of biological materials. As noted before, limiting supplies of biological materials requires that micro scale purification and assay techniques be used. One also needs to consider separation time and temperature when one is dealing with somewhat labile activities. In the current studies, the latter two considerations were not so important.

In this chapter, the results of the preliminary purification and evaluation of techniques is presented. A diagram describing the standard

process of methods evaluation is presented in Figure IV-2. Several different methods were tested (see following section on Preliminary Purification) and the information gained was used to develop a purification scheme for the isolation of antifungal activities from extracts of *Swartzia simplex* seeds. The latter is presented in Chapter V.

Figure IV-2: Standard Procedures for the Evaluation and Development of Preliminary Purification Methods.



Initial characterization of the antifungal activity from *S. simplex*

Heat treatment: Dialyzed neutral extract of *S. simplex* seeds was subjected to a 5-min heat treatment at 50, 60, 70, 80, 90 or 100 °C. After removal of heat-denatured proteins, heat-treated extracts were tested for antifungal activity. Antifungal activity survived heating at 50 °C (Figure IV-3). This fraction retained full activity and completely inhibited conidial germination of *A. flavus*. Antifungal activity was totally lost after heating at 60 °C or higher. The heat sensitivity of the antifungal activity is consistent with the conclusion that the active component is a protein. Heat-treated extracts were also tested for hemagglutination activity. All of the heat-treated extracts retained hemagglutination activity indicating that at least some of the agglutinating activity present in the extracts was not denatured by heat.

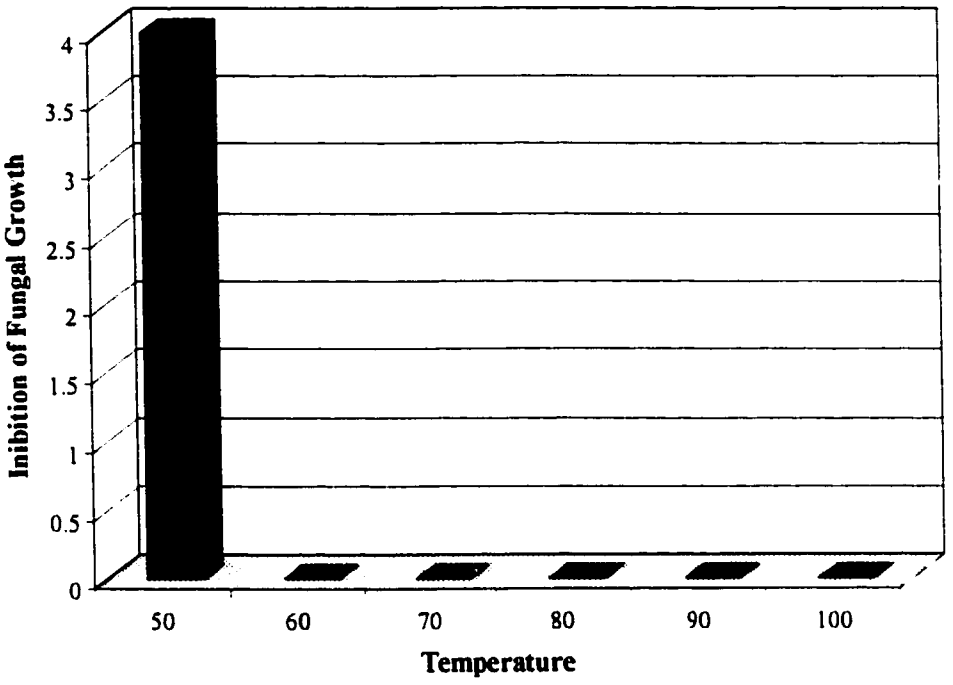


Figure IV-3: Assessment of Antifungal Activity of Heat-Treated Extracts. Dialyzed neutral extracts were heated for 5 min at 50, 60, 70, 80, 90 or 100°C. Heat treated extracts were centrifuged and the supernatants were assayed for antifungal activity against *A. flavus* using the standard bioassay.

Estimation of size by ultrafiltration: Dialyzed neutral extract was concentrated by ultrafiltration using 10, 30, 50 or 100 kDa MWCO concentrators (Centricon). The solution retained after ultrafiltration (i. e. retentate) containing macrosolutes with estimated molecular weights above the membrane cut off and the solution that filtered through the membrane (i. e. filtrate) containing solutes with apparent molecular weights less than the nominal MW cut off were tested for antifungal activity. The retentates from the C10, C30, C50 and C100 concentrators completely inhibited germination of *A. flavus* conidia after 48 h of incubation (Figure IV-4). Moderate to slight inhibition of hyphal growth was observed in all the filtrates in the 48 h assay. When the incubation period was prolonged to 7 days, inhibition of hyphal growth of *A. flavus* by the filtrates was not detectable. However, even after 7 days of incubation, all of the retentates still inhibited germination and growth of this fungus.

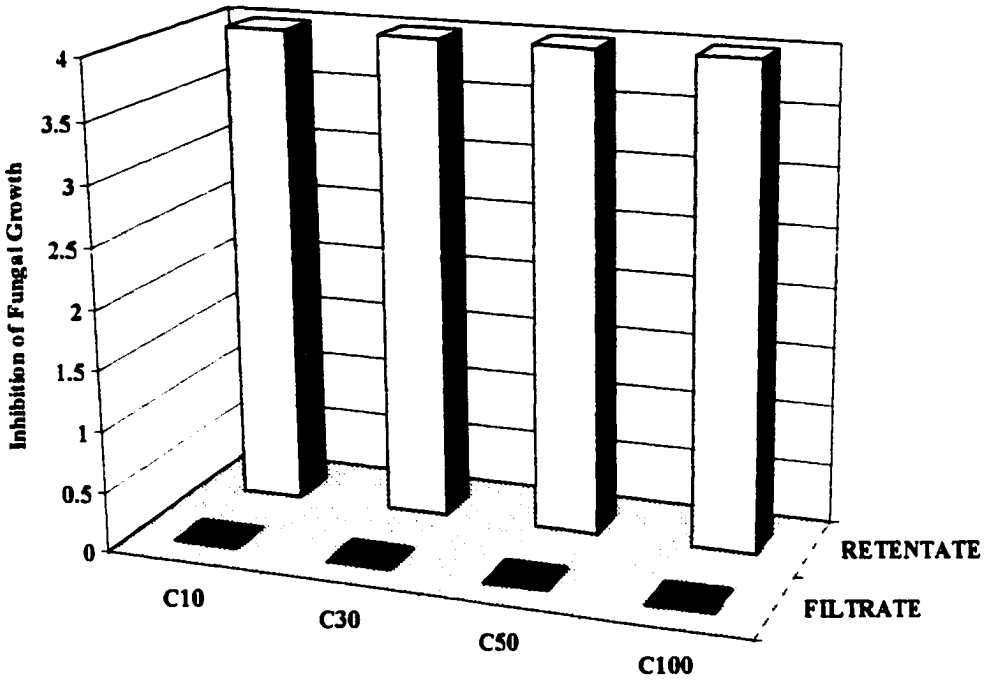


Figure IV-4: Preliminary Size Fractionation of Antifungal Activity. Dialyzed neutral extract was fractionated by ultrafiltration using 10, 30, 50 and 100 kDa MWCO concentrators. One hundred µl of both the retentate and filtrate from each size fractionation were assayed for antifungal activity using *A. flavus*.

Because of the limited heat stability of the bioactive components, heat treatment was not an effective technique for bulk purification. The apparent size of the active components was greater than 3,500 kDa based on the results of dialysis and ultrafiltration. In fact, the activity appears to be greater than 100 kDa in size based on its retention by the C100 concentrator. If this is true, then size-exclusion chromatography may be an effective method of preliminary purification.

Preliminary purification of antifungal activity

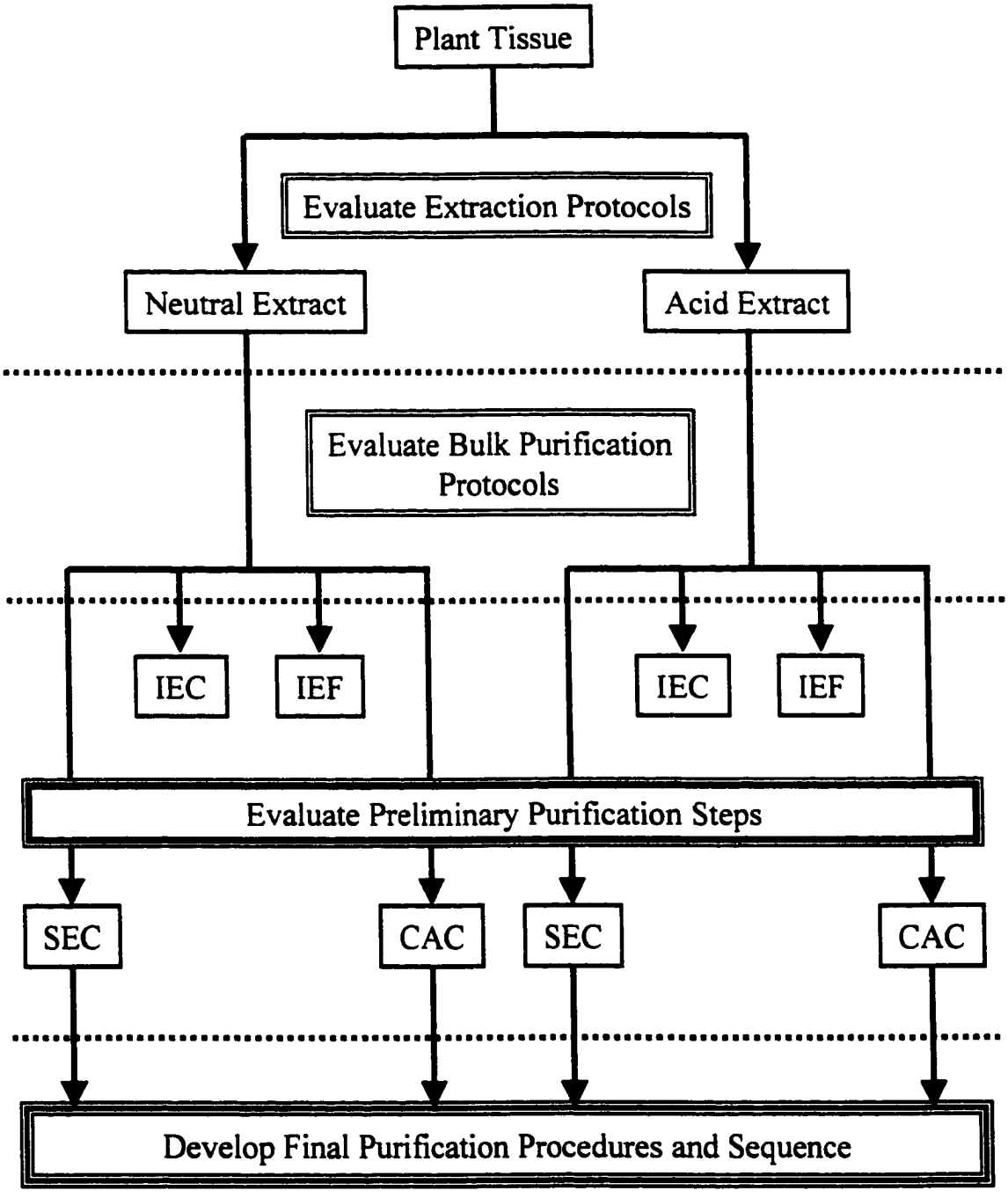
Comparison of extraction procedures: As discussed earlier in this chapter, to develop preliminary bulk methodology for the purification of antifungal activity from *S. simplex* seeds, two different extraction procedures were chosen. In the standard neutral extraction procedure, *S. simplex* seeds were extracted with 10 mM Tris-HCl, pH 8.0, while the second procedure (acid extraction) involved extraction with 0.1 M HCl. The different extraction conditions used may enrich in certain activities, isolate different activities and/or remove contaminating protein. In both cases, the crude extracts were dialyzed to remove low molecular weight factors and tested for antifungal activity. The dialyzed neutral extract provided very strong

inhibition, while the dialyzed acid extract completely prevented fungal growth. Very strong hemagglutination activity was observed in the dialyzed neutral extract. Notably, there was no hemagglutination activity in the dialyzed acid extract suggesting that hemagglutinating activity was sensitive to very low pH. The latter observation is consistent with the conclusion that the agglutinating activity was a protein.

After extraction, various preliminary purification techniques were evaluated for yield and effectiveness. All of these preliminary purification steps (summarized in Figure IV-5) were carried out in parallel with both neutral and acid extracts. These techniques include size-exclusion, chitin-affinity and anion-exchange chromatography, preparative isoelectric focusing and other methods.

Size-exclusion chromatography and chitin-affinity chromatography were the most effective tools for purification of activity from crude extracts. For this reason, results of preliminary fractionation of crude extracts using these two techniques will be discussed first and with the most detail. As discussed earlier, the use of different approaches to purification provides an additional benefit by creating a set of data on the properties of active materials that can help distinguish between different activities. This "triangulation" approach assists in the identification of bioactive proteins.

Figure IV-5: Scheme Outlining the Methods Tested for the Preliminary Purification of Antifungal Proteins from Extracts of *S. simplex* Seeds.



Size-exclusion chromatography

Neutral Extract: To begin, 500 µl of dialyzed neutral extract was fractionated by size-exclusion chromatography (SEC) on a Pharmacia Superose 12 column. According to the absorbance profile (280 nm), the majority of the 280-absorbing material was in fractions 2-5 with smaller peaks in fractions 8 and 11 (Figure IV-6). The middle peak exhibited a characteristic shoulder in fractions 9 and 10. All 20 fractions obtained from the Superose column were assayed for antifungal activity. The results of the bioassay revealed that fraction 9 and 10 very strongly inhibited growth of *A. flavus*, while fraction 1 and 4 completely inhibited conidial germination and growth (Figure IV-7). Only slight inhibition of hyphal growth was observed in fractions 12, 13 and 15.

Fractions 9 and 10 agglutinated human and rabbit red blood cells (Figure IV-8). The strong agglutination reaction and inhibition of fungal growth by these fractions suggest that there may be a correlation between hemagglutinating activity and antifungal activity. Chitinase and glucanase activities in the Superose 12 fractions were also measured. According to the results of the chitinase colorimetric assay, the main peak of chitinase activity

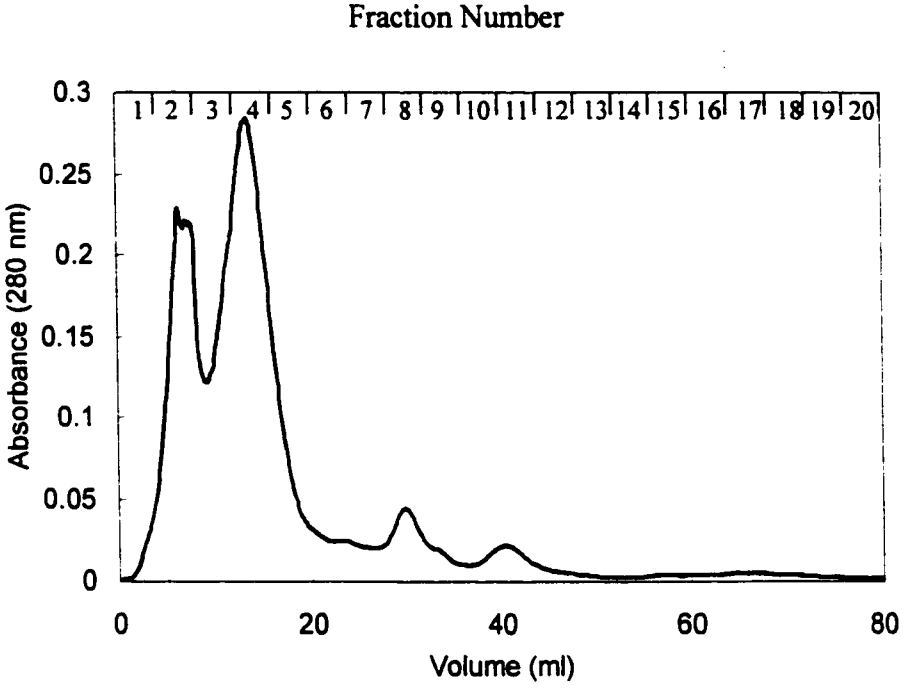


Figure IV-6: Absorbance Profile of Neutral Extract Fractionated by Size Exclusion Chromatography. Five hundred µl of concentrated (2x) neutral extract was applied to the column. Proteins were eluted with 10 mM Tris-HCl, pH 8.0.

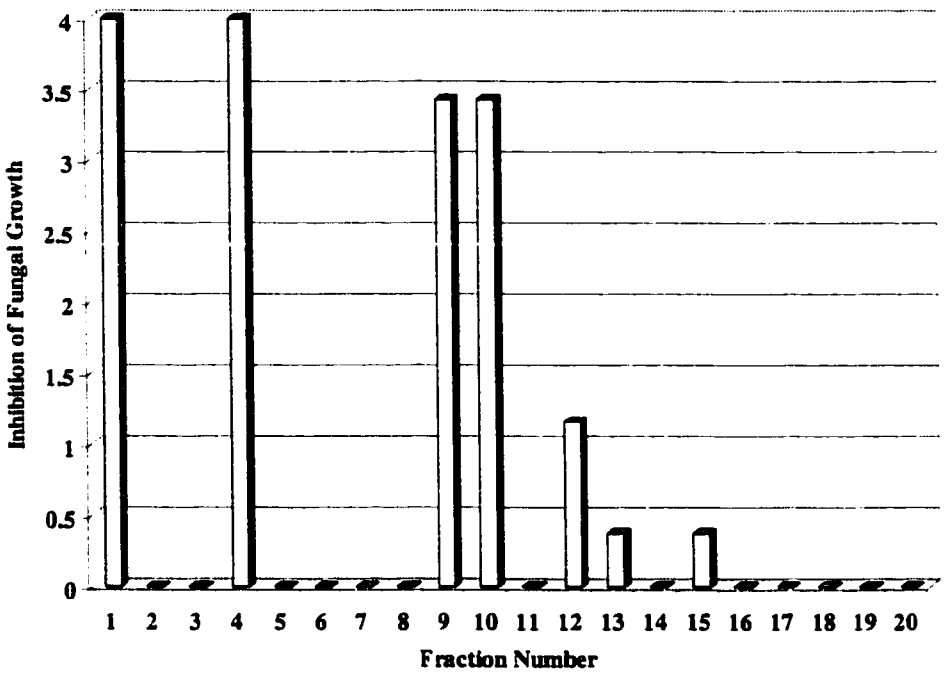


Figure IV-7: Antifungal Activity of Fractions From Neutral Extract Superose 12 Column. Neutral extract was separated by size exclusion chromatography on a Superose 12 column. A sample (100 µl) from each fraction was assayed for antifungal activity using *A. flavus.*

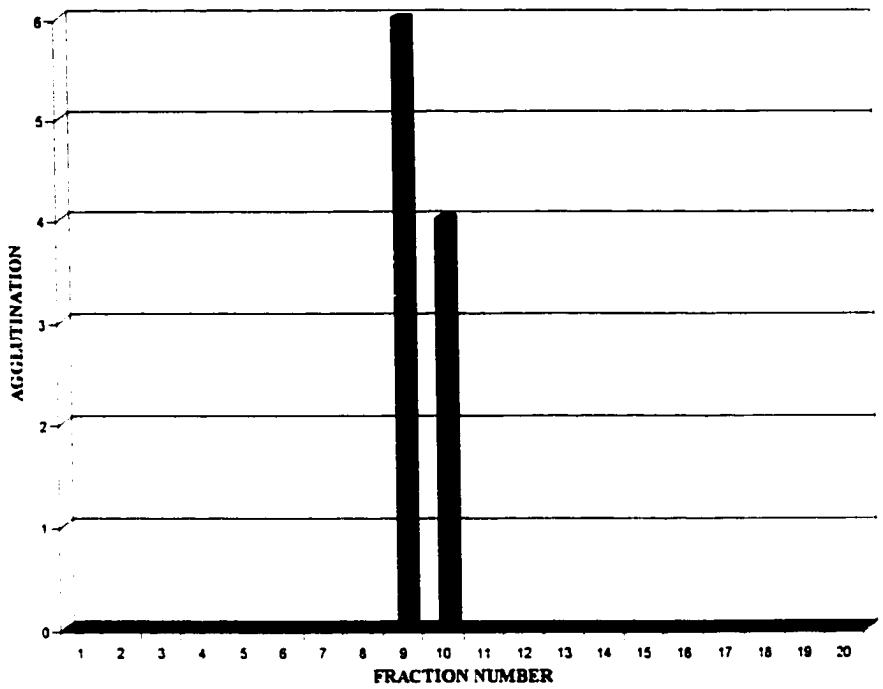


Figure IV-8: Hemagglutination of Fractions from Neutral Extract Fractionated by Size Exclusion Chromatography on a Superose 12 Column. A sample (100 µl) from each fraction was tested for agglutinating activity with human red blood cells.

was present in fractions 4 to 5 (Figure IV-9). Fractions 2 to 4 had the highest glucanase activity, although there was a trace of activity in all 20 fractions (Figure IV-10).

The protein banding patterns of the neutral extract Superose 12 fractions were analyzed by SDS-PAGE (Figure IV-11). This analysis revealed that fraction 1 contained 5-6 minor bands. A number of heavily stained bands with estimated molecular weights between 28 to 35 kDa were present in fractions containing chitinase (fractions 2-6), glucanase (fractions 1-4) and antifungal activity (fraction 4). The molecular weights of these proteins are similar to the molecular weights of many plant chitinases and glucanases. Fraction 4 that had very strong antifungal activity, the highest glucanase activity and very good chitinase activity contained several other prominent bands with estimated molecular weights of approximately 25, 18 and 8 kDa. Fraction 9 and 10 exhibited 2 major bands of approximately 21 to 22 kDa. Bands with similar molecular weights were also present in fractions 3-17 but the intensity of staining of these bands was the highest in fractions 9 and 10 and was relatively low in fractions 3-5. Fraction 9 also contained four distinct bands with molecular weights from 31 kDa to 66 kDa along with some minor bands including several diffuse bands from 10 to 16 kDa.

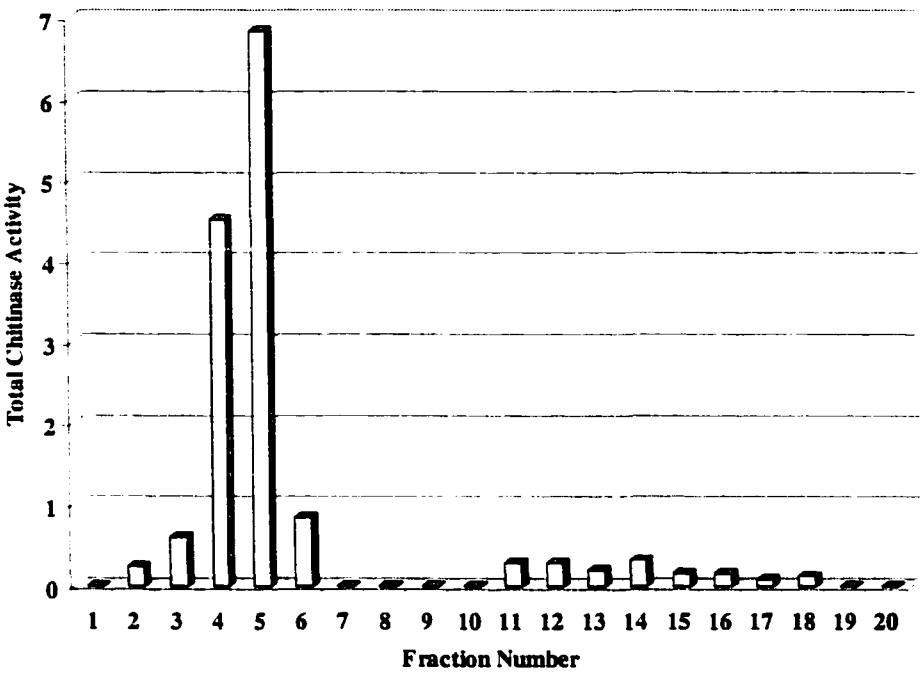


Figure IV-9: Total Chitinase Activity of Fractions from Neutral Extract Superose 12 Column. A 100 µl sample from each fraction was used for the assay . Chitinase activity in each fraction was measured using the colorimetric chitinase assay.

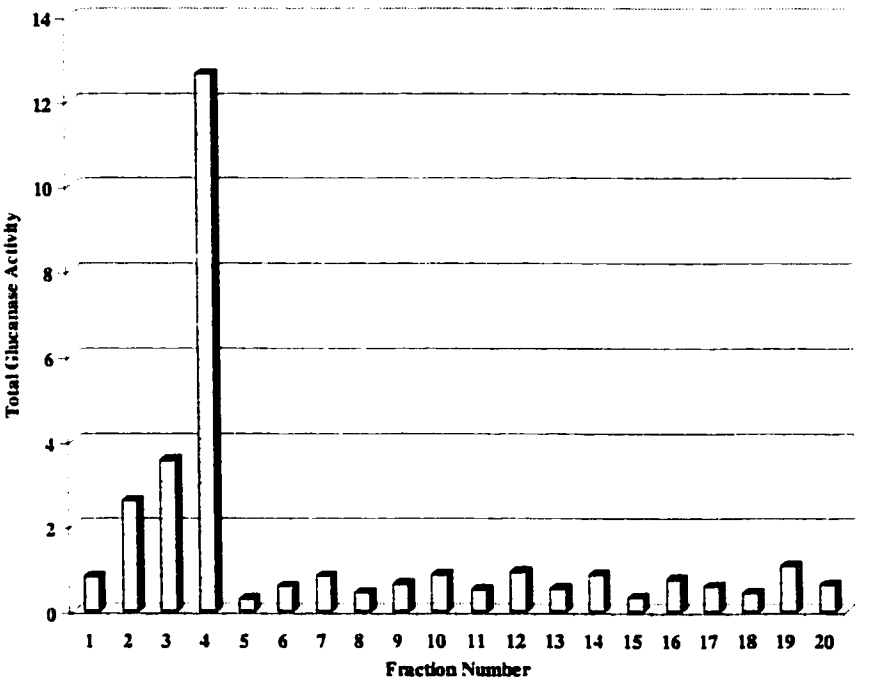


Figure IV-10: Total Glucanase Activity of Fractions after Superose 12 Chromatography of Neutral Extract. A portion of each fraction was concentrated (2x) and 100 µl of the concentrated sample was evaluated for glucanase activity using a colorimetric assay.

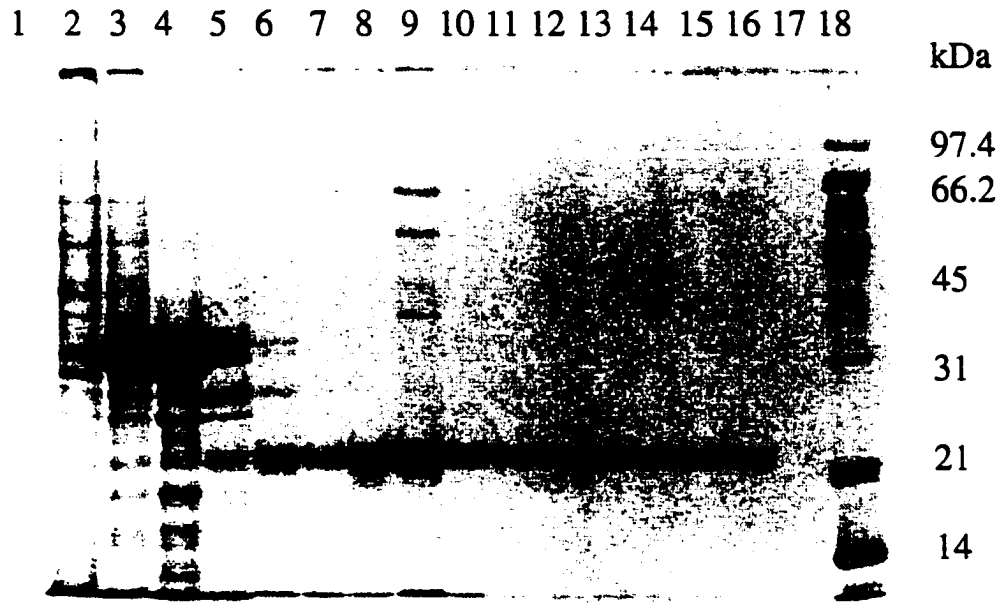


Figure IV-11: SDS-PAGE Gel of Neutral Extract Superose 12 Fractions. One hundred µl from each fraction was dried *in vacuo*, resuspended in 10 µl of sample buffer and boiled for 5 min before loading onto the gel. Lane 1 to 17, Superose 12 fractions 1 to 17. Lane 18, molecular weight markers.

Acid extract: Dialyzed acid extract (0.5 ml) was fractionated by size-exclusion chromatography using the same materials and methods as used for the neutral extract. The absorbance pattern (Figure IV-12) was substantially different from the profile for the neutral extract. For instance, there was a broad peak of 280 absorbance in fractions 2-7 but individual peaks were not clearly resolved. Additional 280-absorbing material was eluted in fractions 8-20. These late-eluting constituents gave broad, non-resolved peaks.

Incorporation of fractions 3-5 in the fungal bioassay resulted in complete inhibition of conidial germination and fungal growth (Figure IV-13). Unlike results obtained from the neutral extract Superose 12 fractions, no antifungal activity was found in fractions 9 and 10. The absence of antifungal activity in fractions 9 and 10 support the conclusion that there are differences in the active components found in the acid and the neutral extracts. When tested, none of the acid-extract Superose 12 fractions agglutinated human or rabbit red blood cells. Again, the apparent loss of both antifungal activity and agglutination activity in fractions 9 and 10 after size-exclusion chromatography of the acid extract adds support to the notion that at least some of the antifungal factors present in the neutral extract may be agglutinins. Chitinase and glucanase activity was present in fractions

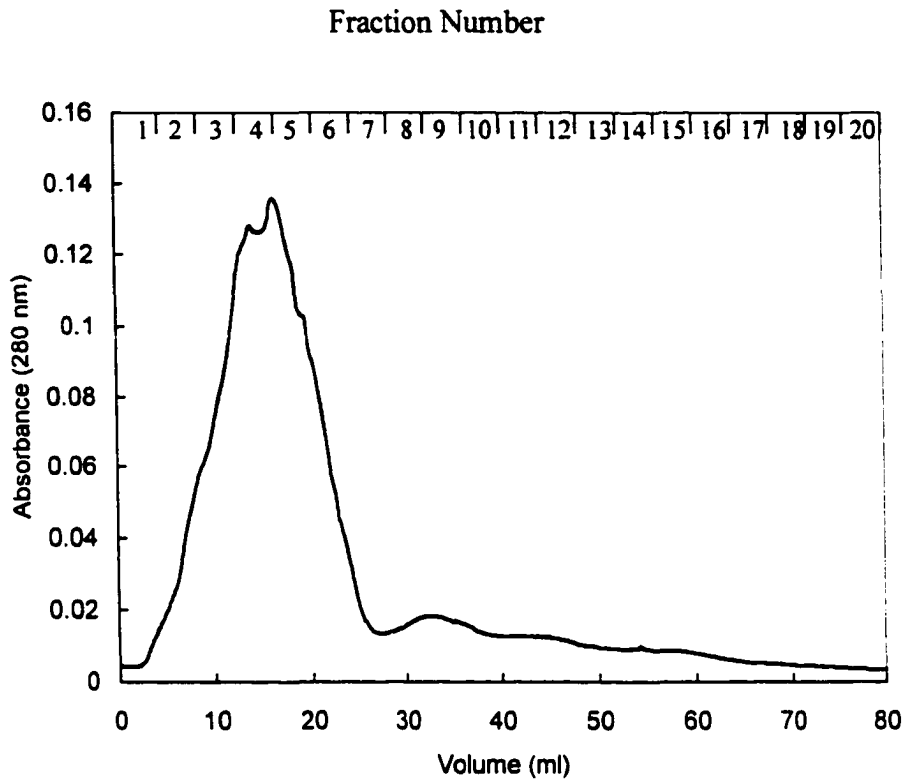


Figure IV-12: Absorbance Profile of Acid Extract Separated by Size Exclusion Chromatography. Five hundred µl of the concentrated (2x) acid extract was loaded onto the column. Proteins were eluted in 10 mM Tris-HCl, pH 8.0.

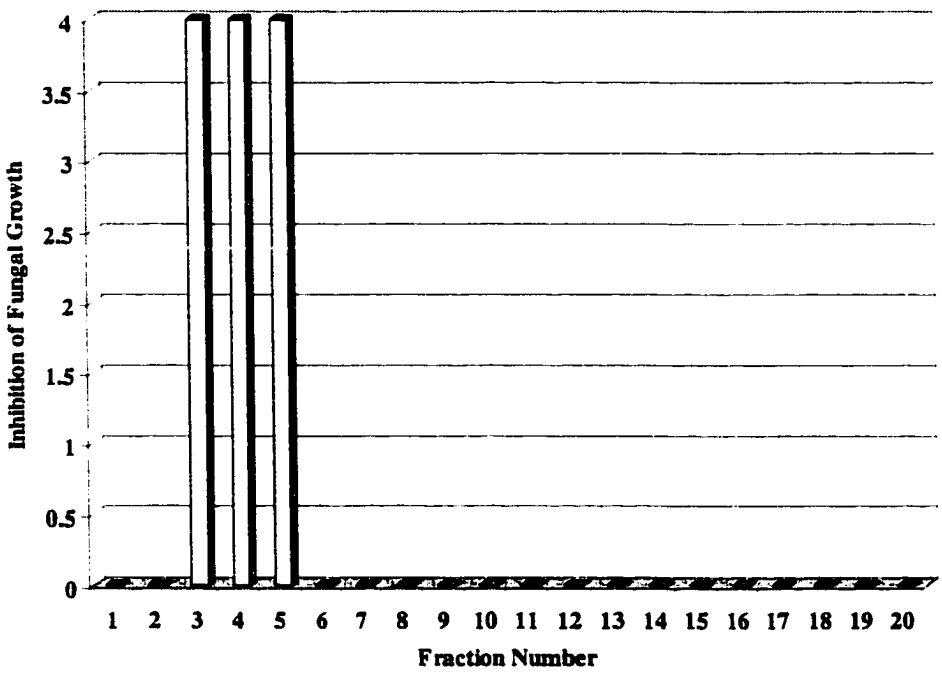


Figure IV-13: Antifungal Activity of Fractions from Acid Extract Superose 12 Column. Acid extract was separated on a Superose 12. A sample (100 µl) from each fraction was assayed for antifungal activity using *A. flavus*.

2-5 (Figure IV-14) and 2-5 (data not shown), respectively.

SDS-PAGE analysis of acid extract Superose 12 fractions revealed several protein bands in fractions 3 to 7 including two prominent bands with approximate molecular weights between 30 and 32 kDa (Figure IV-15). Two distinct bands between 14 and 21 kDa in size were present in all the fractions. Several additional bands between 21 and 14 kDa were present in fractions 3 to 6 only.

In an attempt to determine the number of individual proteins present in the active fractions, fraction 5 from the acid extract Superose 12 column was further purified by reverse phase HPLC at the Molecular Biology Resource Center at the University of Oklahoma Health Sciences Center (OU HSC). Approximately 12 different peaks were detected based on the absorbance profile at 218 nm (Figure IV-16). The fractions from the micro-HPLC were dried and subjected to SDS-PAGE. The results of a silver-stained SDS gel of these fractions are presented in Figure IV-17. Although the pattern of protein bands differed in each fraction, the major peaks from HPLC fractions 10-19 contained one or two major bands with very similar molecular weights in the range of 28 to 31 kDa.

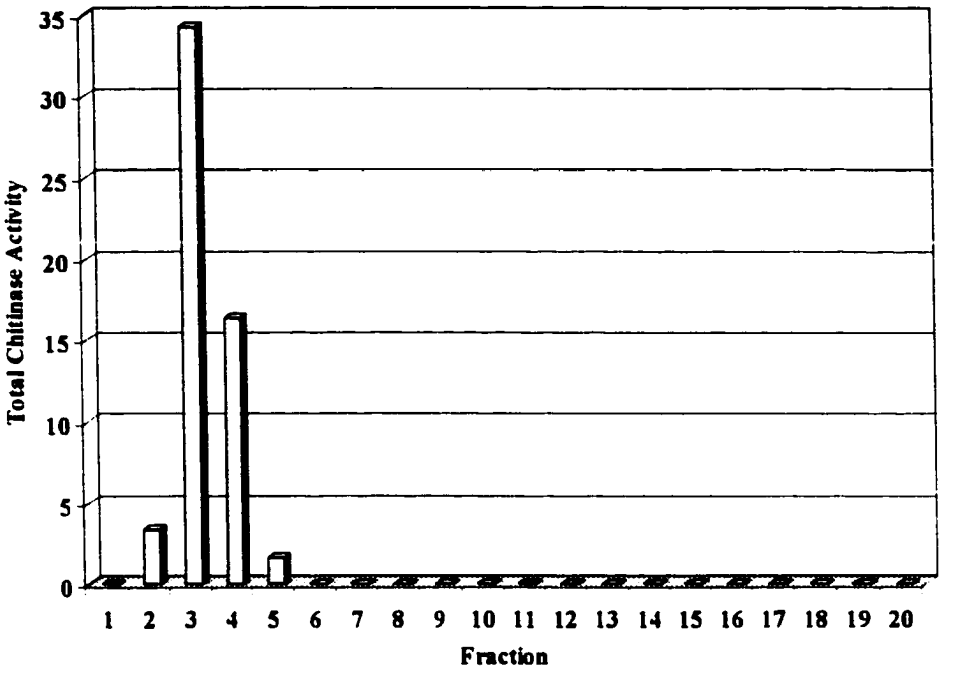


Figure IV-14: Total Chitinase Activity of Acid Extract Fractionated by Size Exclusion Chromatography on a Superose 12 Column. A sample (100 µl) from each fraction was used foe the assay. Chitinase activity in each fraction was measured using the colorimetric chitinase assay.

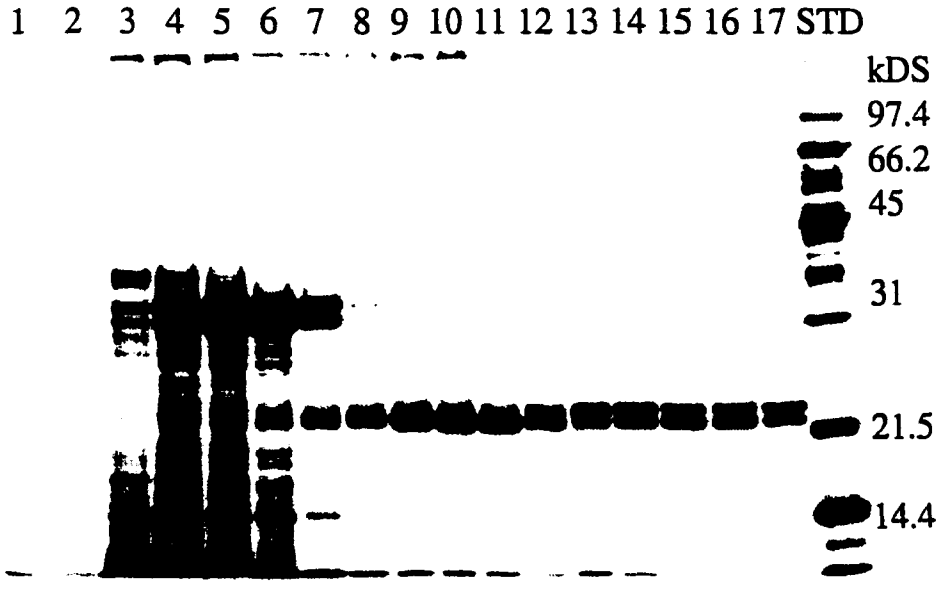
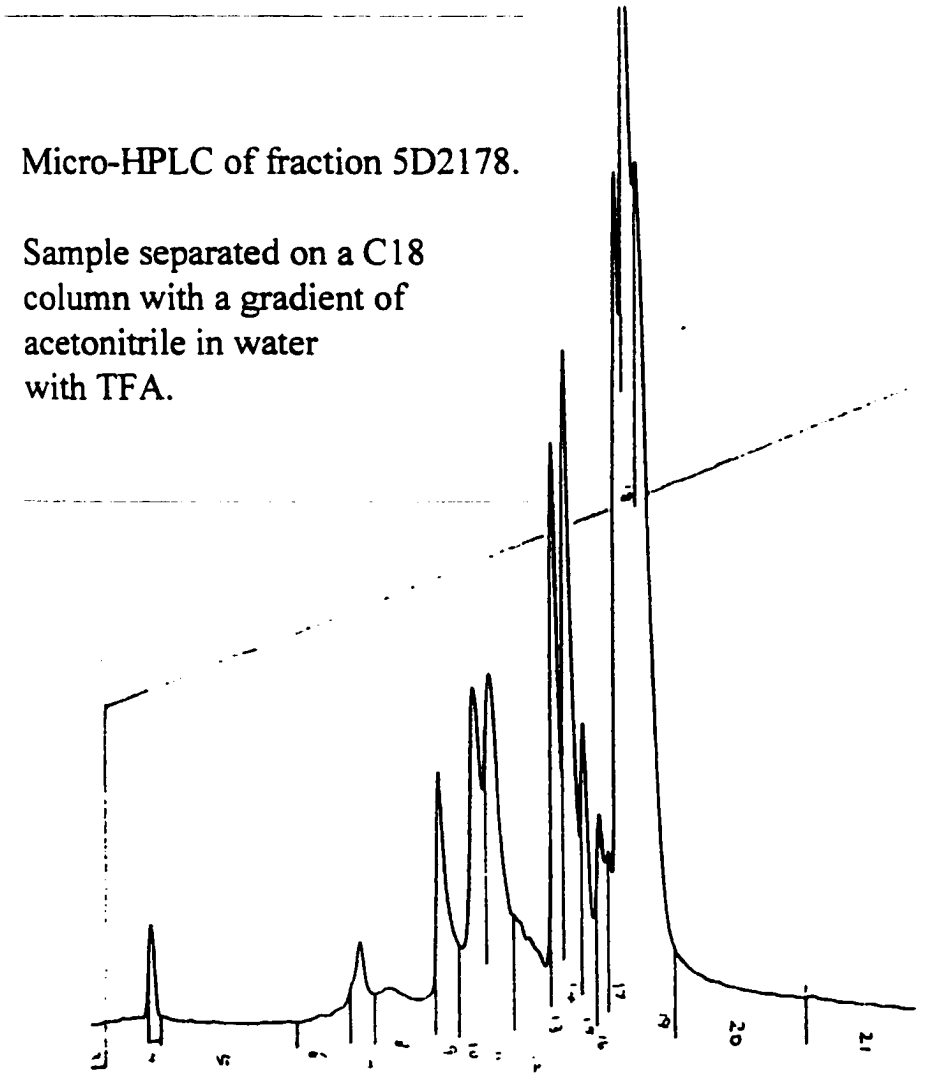


Figure IV-15: SDS-PAGE Gel of Acid Extract Separated on a Superose 12 Column. One hundred µl of each fraction was dried *in vacuo*, resuspended in 10 µl of sample buffer and boiled for 5 min before loading onto the gel. Lane 1 to 17, Superose 12 fractions 1 to 17.

Figure V-16: HPLC Profile of Acid Extract Superose 12 Fraction 5.



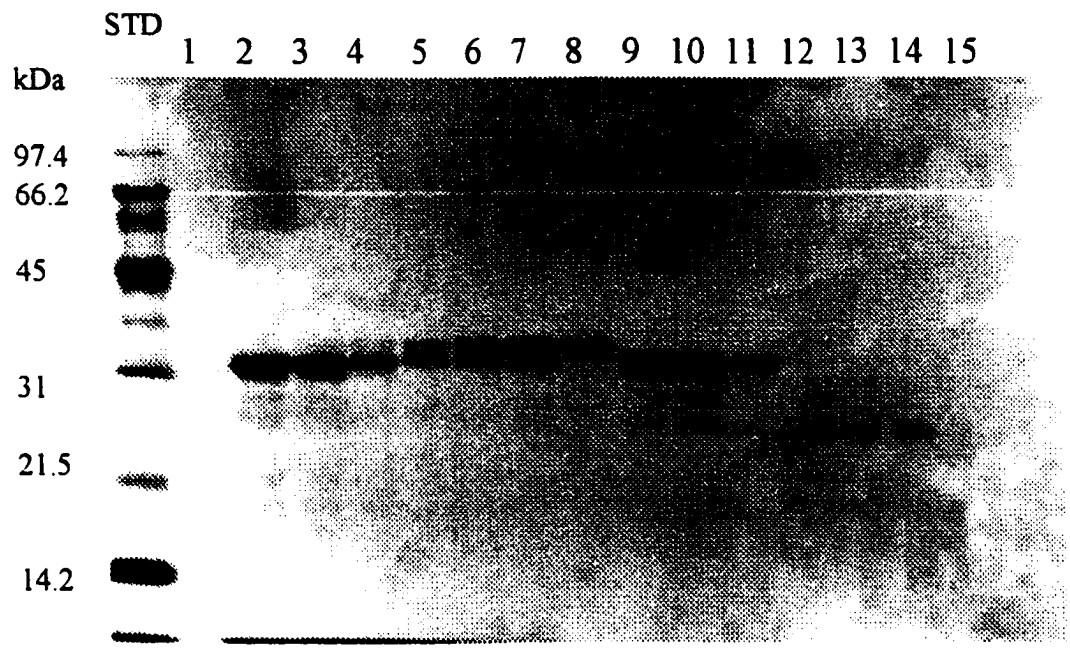


Figure IV-17: SDS-PAGE Gel of HPLC Column Fractions. Acid extract was separated on a Superose 12 column. Fraction 5 was further fractionated by HPLC. Individual fractions were dried and separated on a 12.5% SDS gel. The gel was silver stained to detect protein bands. Lane 1, molecular weight standard. Lane 2 to 15, HPLC fraction 4 to 19.

Affinity chromatography

Chitin-affinity chromatography has been used effectively to isolate chitin-binding antifungal proteins. Chitin-affinity chromatography was evaluated as a preliminary purification method. Dialyzed neutral extract and acid extract were both fractionated by chitin-affinity chromatography.

Neutral chitin-affinity chromatography: Two different elution procedures were used to fractionate dialyzed neutral extract on the chitin-affinity column. Once the sample was applied to the chitin column, bound components were eluted stepwise with either acetic acid/sodium hydroxide or NaCl. In the case of the low and high pH elution, 5 ml of dialyzed neutral extract was routinely loaded onto the column equilibrated with 10 mM Tris-HCl, pH 8.0. The flow through was collected and the unbound proteins were removed first with 100 ml of 10 mM Tris-HCl, pH 8.0 (first Tris wash) followed by washes with 0.1 M NaCl and 1 M NaCl in 20 mM sodium acetate buffer, pH 5.6.

After washing the column, bound proteins were eluted first with 100 ml of 0.1 M acetic acid followed by 100 ml of 0.1 M NaOH as described in the Materials and Methods. The elution profiles for the acetic acid and

sodium hydroxide steps are shown in Figure IV-18. The majority of the 280-absorbing material released from the column was eluted with 0.1 M acetic acid and the remainder was eluted as a broad peak with 0.1 M NaOH. All of the fractions except the first Tris wash, second Tris wash and the supernatant after ammonium sulfate precipitation of the NaOH eluate strongly inhibited fungal growth (Figure IV-19). Chitinase and glucanase activities (Figure IV-20 and IV-21, respectively) were present only in the flow through and the resuspended pellets after ammonium sulfate precipitation of the acetic acid and NaOH eluates. The presence of chitinases and glucanases in both the high and the low pH eluates suggested that there were different forms of chitinase and glucanase present in the neutral extract. This suggestion was confirmed using the chitinase and glucanase in-gel assay. Multiple forms of both activities were detected in the neutral extract and in different chitin column fractions. These results will be presented in greater detail in Chapter V.

All of the fractions from the chitin column were assayed for hemagglutination (Figure IV-22). Before analysis, the flow through, Tris washes, 0.1 M NaCl wash and 1 M NaCl wash were concentrated using a Centricon C10 (10,000 MWCO) concentrator. For the hemagglutination assay, 100 µl of each of the concentrated retentates along with an equal

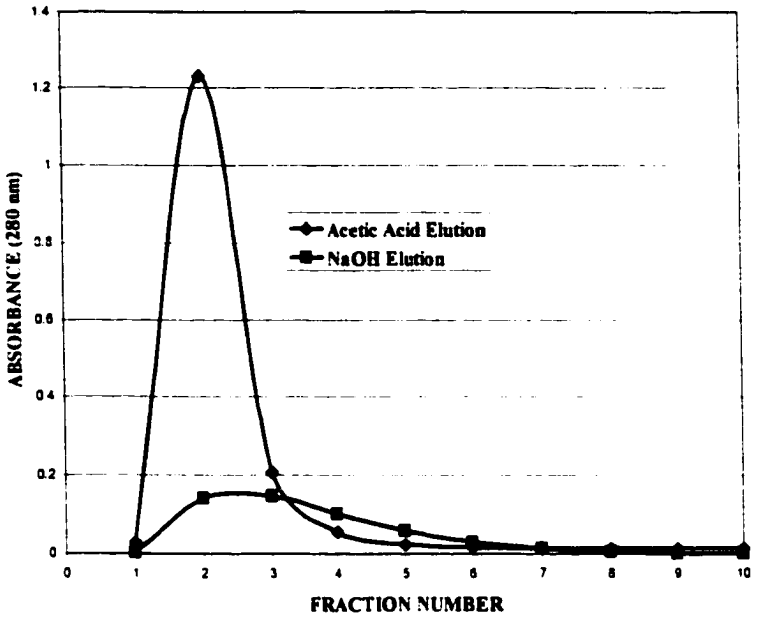


Figure IV-18: Absorbance Profile of Acetic Acid and Sodium Hydroxide Elution of Chitin-affinity Column. Neutral extract was applied to the chitin-affinity column and eluted with 0.1 M acetic acid followed by elution with 0.1 M sodium hydroxide. Fractions (10 ml) were collected and the absorbance at 280 nm was measured.

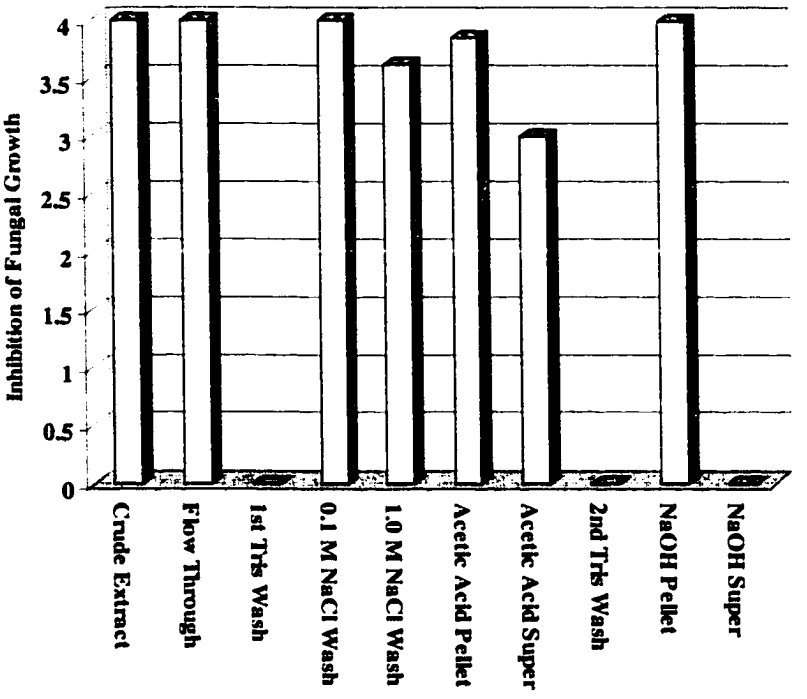


Figure IV-19: Antifungal Activity of Neutral Chitin Column Fractions. Five ml of dialyzed neutral extract was fractionated on a chitin affinity column. Fractions from the chitin column were dialyzed against 10 mM Tris-HCl, pH, 8.0, and 100 µl from each fraction was assayed for antifungal activity using *A. flavus*.

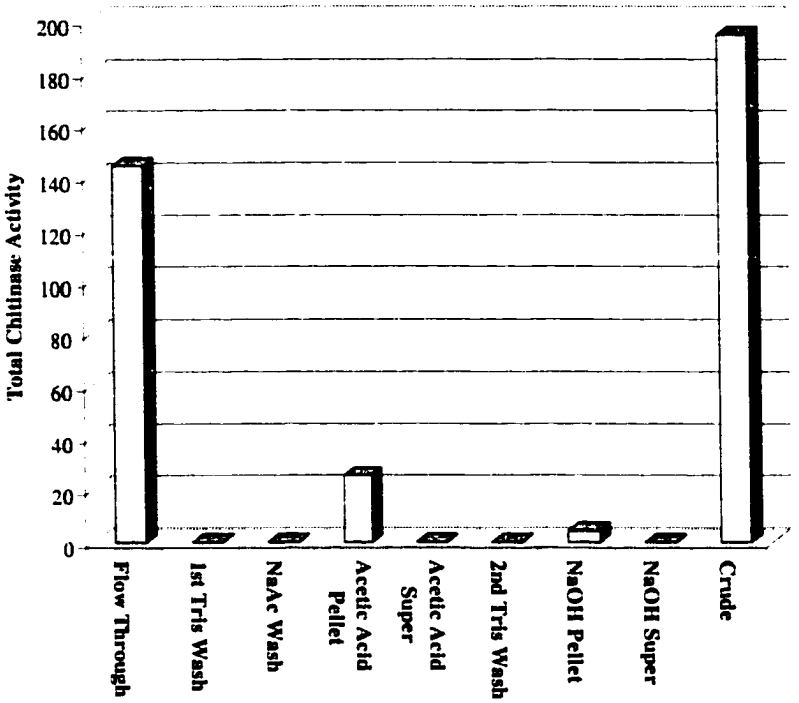


Figure IV-20: Total Chitinase Activity of Neutral Chitin Column Fractions. Chitinase activity was measured using the colorimetric chitinase assay. A sample (100 µl) from each fraction was used in the standard assay.

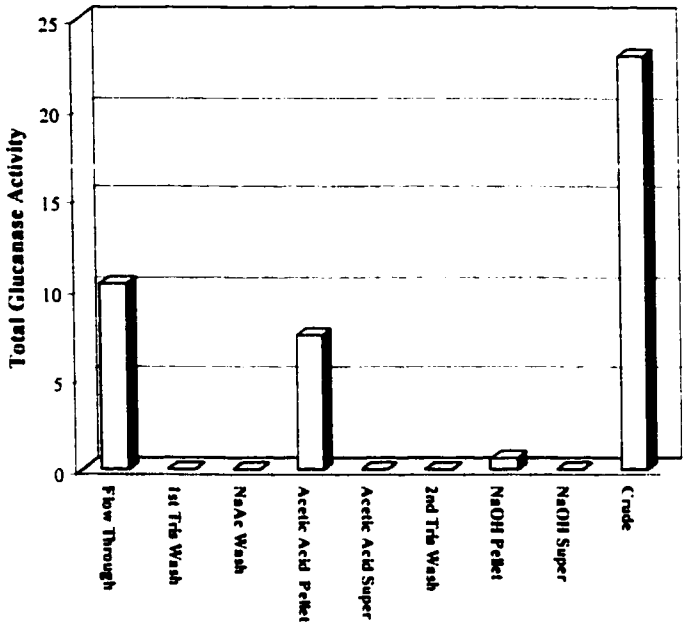


Figure IV-21: Total Glucanase Activity of Neutral Chitin Column Fractions. A sample of each fraction was concentrated (2x). One hundred µl of concentrated sample from each fraction was evaluated for glucanase activity using the colorimetric glucanase assay.

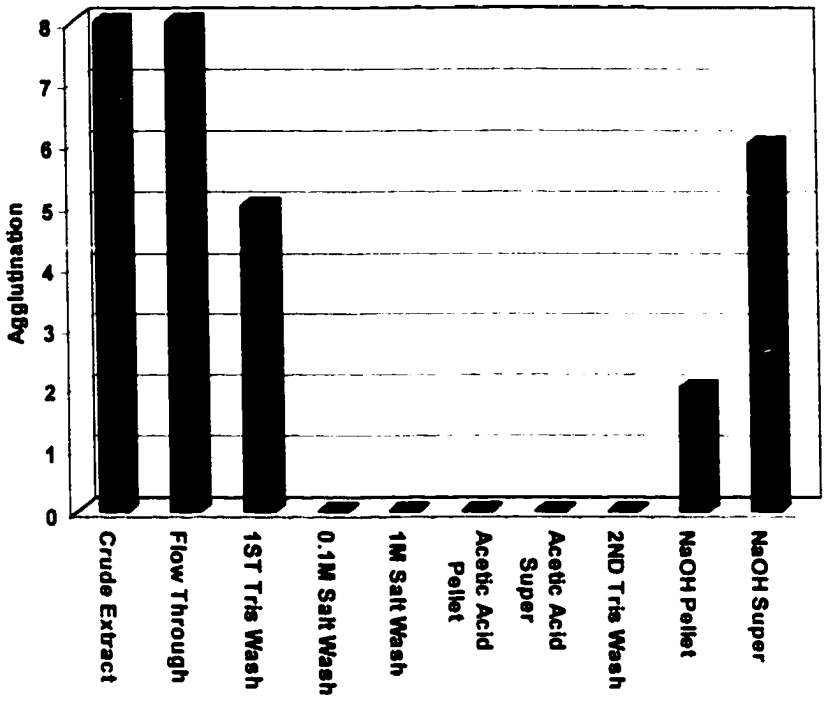


Figure IV-22: Hemagglutination of Fractions from the Neutral Extract Chitin Column. Neutral extract was fractionated on a chitin affinity column. A sample of each fraction except acetic acid eluate and sodium hydroxide eluate were concentrated (2x). One hundred µl of concentrated samples from each fraction and one hundred µl of neutral extract, acetic acid eluate and sodium hydroxide eluate each were tested for hemagglutinating activity using human red blood cells.

volume of the resuspended and dialyzed pellets after ammonium sulfate precipitation of the acetic acid and NaOH eluates were tested. Different forms of agglutinin were present in the neutral extract. A portion of the agglutinating activity did not bind to the chitin column. This material, the non-chitin binding fraction, was found in the flow through and the first Tris wash. Agglutinins that did bind to the chitin column were eluted at high pH. Only a small amount of this agglutinating activity was precipitated by ammonium sulfate, while most of the agglutinating activity remained soluble in the supernatant.

The occurrence of enzymatic activity, antifungal activity and agglutinating activity in the flow through and initial washes may indicate that the column was overloaded. Alternatively, different isoforms of an activity may be present in different fractions. For instance, some isoforms of chitinase may bind to the column while others do not bind. In experiments in which larger volumes of extract were applied to the same size column, the relative percentage of the total activity loaded onto the column that did not bind remained the same. This observation suggests that the column was not overloaded. Therefore, molecular differences or

interactions between individual proteins may account for the lack of binding of certain forms.

SDS-PAGE analysis of the chitin column fractions revealed a number of protein bands with molecular weights between 14 and 45 kDa (Figure IV-23). Seven prominent bands were visible in the lane containing flow through. Out of the seven distinct bands, there were two major bands with molecular weights of approximately 31-33 kDa. Two bands of similar size were the major proteins in the acetic acid and NaOH eluates and were also present to some extent in the 1st Tris wash and 1 M NaCl wash. Two other bands with molecular weights between 21 and 31 kDa were seen in the flow through, 1 M NaCl wash and acetic acid eluate. A 45-kDa protein was present in the flow through and acetic acid eluate. The sodium hydroxide pellet was very clean and only two bands with molecular weights about 31 kDa were detected in this fraction. There were no visible bands in the lane containing the NaOH supernatant.

Although bands of similar size were detected in different fractions, the absolute identity of individual proteins requires further investigation. Since concentrated samples were not used for the SDS gel, it is possible that any protein responsible for the agglutinating activity present in the concentrated

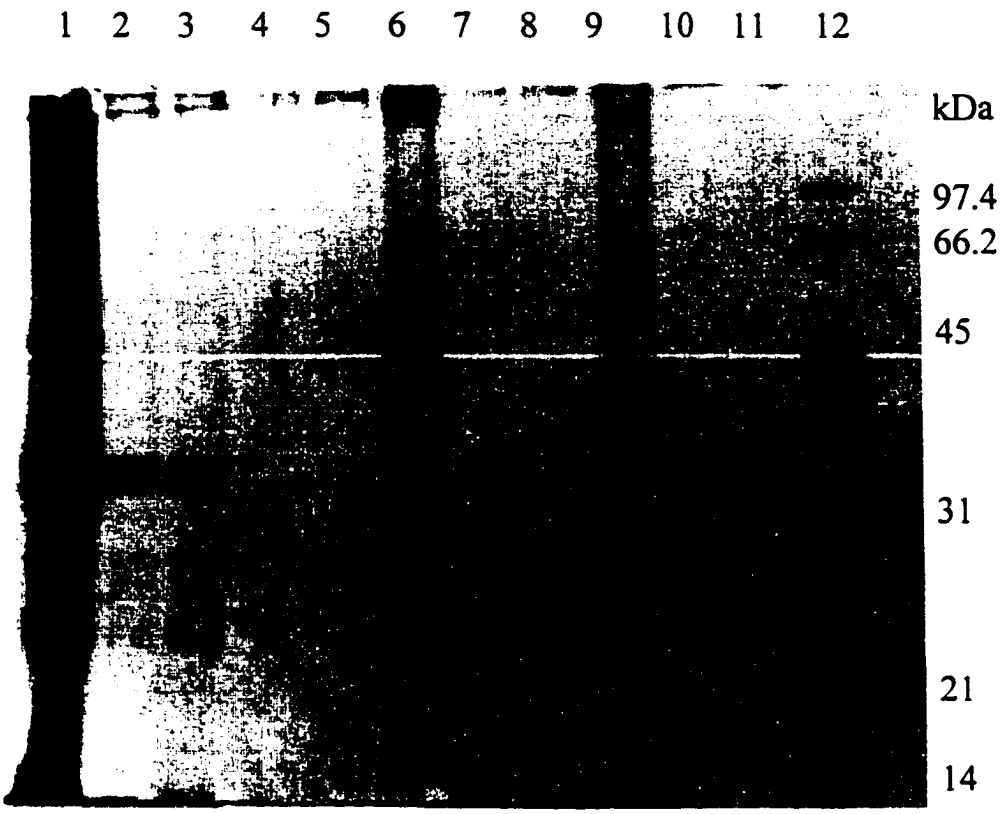


Figure IV-23: SDS-PAGE Gel of Neutral Chitin Column Fractions. A sample (100 µl) of each fraction was dried *in vacuo*, resuspended in 10 µl of sample buffer and boiled for 5 min before loading onto the gel. Lane 1, neutral extract; lane 2, flow through; lane 3, 1st Tris wash; lane 4, 0.1 M salt wash; lane 5, 1 M salt wash; lane 6, acetic acid eluate; lane 7, acetic acid supernatant; lane7, 2nd Tris wash; lane 8, 2nd 1 M salt wash; lane 9, sodium hydroxide eluate; lane 10, sodium hydroxide supernatant; lane 12, molecular weight markers.

retentate from the NaOH supernatant would not have been visible in the SDS gel.

For the salt elution, the column was washed first with 10 mM Tris-HCl, pH 8.0 to remove unbound proteins. Subsequently, bound proteins were eluted stepwise with 100 ml each of 0.1 M, 0.2 M, 0.3 M, 0.4 M, 0.5 M and 1 M NaCl in 10 mM Tris-HCl, pH 8.0. Very strong antifungal activity was present in the flow through and first Tris wash (data not shown). Agglutinating activity was present in the same fractions but not in the fractions eluted with salt (data not shown). There was little difference between the protein-banding pattern of the crude extract applied to the column and the flow through containing the antifungal activity (data not shown). Because the only antifungal activity detected under these conditions was primarily in the flow through and not in the fractions eluted with salt, this method was not used for further purification. This flow through, however, may be very useful starting material for further purification of antifungal activity and agglutinin that did not bind tightly to the column. Presumably, other activities that could be eluted with stronger eluants such as acetic acid remained tightly bound to the column. Alternatively, chromatographic conditions could be modified to achieve better separation of specific activities.

Acid chitin column chromatography: Two slightly different acid extraction procedures were used to prepare acid extract for chitin-affinity chromatography. To begin, 10 g of *S. simplex* seeds were homogenized in 0.1 N HCl according to the methods described by Peumans and associates. (Peumans et al., 1983). Following homogenization, the extract was centrifuged at 20,000 x g for 10 min. The clear supernatant was saved and the pellet containing cell debris and denatured protein was discarded. The pH of the crude extract was adjusted to 3.8 with 2 N NaOH and allowed to stand on ice for one h. At the end of this time, the cloudy supernatant was centrifuged (20,000 x g for 10 min) to remove the denatured protein and the clear supernatant was filtered through Whatman 3MM filter paper. The filtrate i. e. crude neutralized acid extract (5 ml) was loaded onto a chitin column (equilibrated with 50 mM NaAc buffer, pH 3.8) and fractionated on the column according to the procedures described in the materials and methods section. An aliquot of the neutralized extract and all the fractions from the neutralized chitin column were dialyzed in 10 mM Tris-HCl, pH, 8.0, and assayed for antifungal activity.

Antifungal activity was not detected in the dialyzed neutralized acid extract or in any of the fractions from the neutralized chitin column. Apparently, antifungal activity did not survive the acid extraction followed

by the neutralization process used in this particular procedure. All of the agglutination activity was also lost during extraction and neutralization.

Although antifungal activity did not survive the combination of acid extraction followed by neutralization with base, activity was retained using a modification of the original acid extraction and neutralization procedure used by Peumans and co-workers. After extraction with 0.1 N HCl, the tissue was centrifuged (15,000 x g; 15 min) and the resulting supernatant was not neutralized with NaOH as described above. Instead, the supernatant was immediately dialyzed against 10 mM Tris-HCl (pH 8.0). Dialysis buffer was changed every 4 to 6 h over a 72 h period. The dialyzed acid extract was tested for antifungal as well as agglutination activity. As observed previously, agglutination activity did not survive acid extraction. Antifungal activity, however, was not denatured in this process. Dialyzed acid extract completely inhibited *A. flavus* conidial germination in the 48 h assay and continued to inhibit growth even after prolonged incubation for 7 days. Apparently, the process of extraction with 0.1 N HCl did not destroy antifungal activity but activity was lost when the acid extract was neutralized with NaOH.

On this basis, *S. simplex* dialyzed acid extract was used from this point for preliminary fractionation by chitin-affinity chromatography. The

chitin column in this case was equilibrated with 20 mM sodium acetate (pH 5.6) instead of 50 mM sodium acetate (pH 3.8) as used for neutralized chitin column. As done previously, 5 ml of dialyzed acid extract was loaded onto the column, the flow through was collected and the column was washed with column equilibration buffer, buffer plus 0.1 M NaCl and buffer plus 1.0 M NaCl. Bound proteins were eluted with 0.1 M acetic acid and 0.1 M NaOH as described in the Materials and Methods. The amount of protein eluted with acetic acid, as measured by the absorbance profile at 280 nm, was much lower than the amount of protein eluted from the neutral chitin column with acetic acid (data not shown). Antifungal activity was detected in the flow through, acetic acid eluate and sodium hydroxide eluate (Figure IV-24). Complete inhibition of *A. flavus* conidial germination was observed in wells containing the flow through. The acetic acid eluate (dialyzed ammonium sulfate pellet) showed very strong inhibition and the sodium hydroxide eluate (dialyzed AS pellet) exhibited moderate inhibition of hyphal growth.

Chitinases and glucanases were detected in the flow through, acetic acid and NaOH eluates (Figure IV-25 and IV-26). However, the total chitinase and glucanase activities recovered in the acid chitin column fractions were substantially lower than the total chitinase and glucanase activity found in the neutral chitin column fractions.

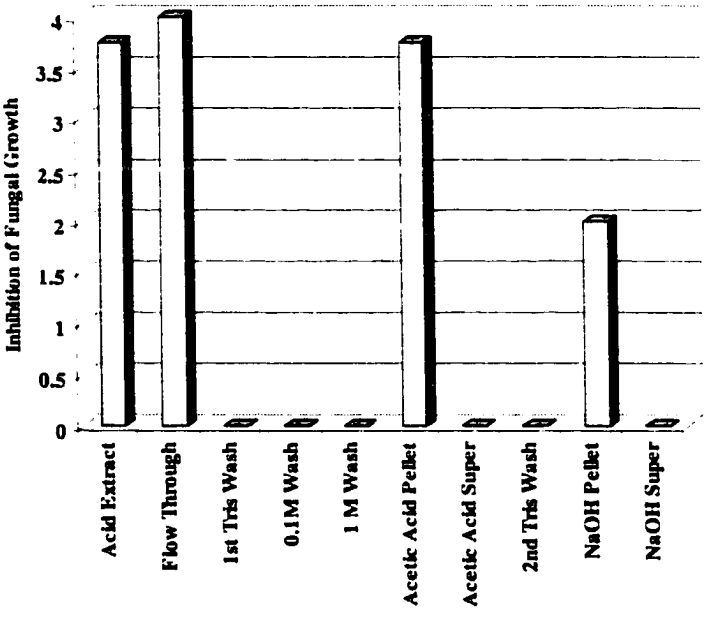


Figure IV-24: Antifungal Activity of Acid Chitin Column Fractions. Five ml of dialyzed acid extract was fractionated on a chitin affinity column. Fractions from the chitin column were dialyzed against 10 mM Tris, pH, 8.0, and 100 µl from each fraction was assayed for antifungal activity using *A. flavus*. Samples from all other fractions except acid extract, acetic acid eluate and sodium hydroxide eluate were concentrated (2x) before assay.

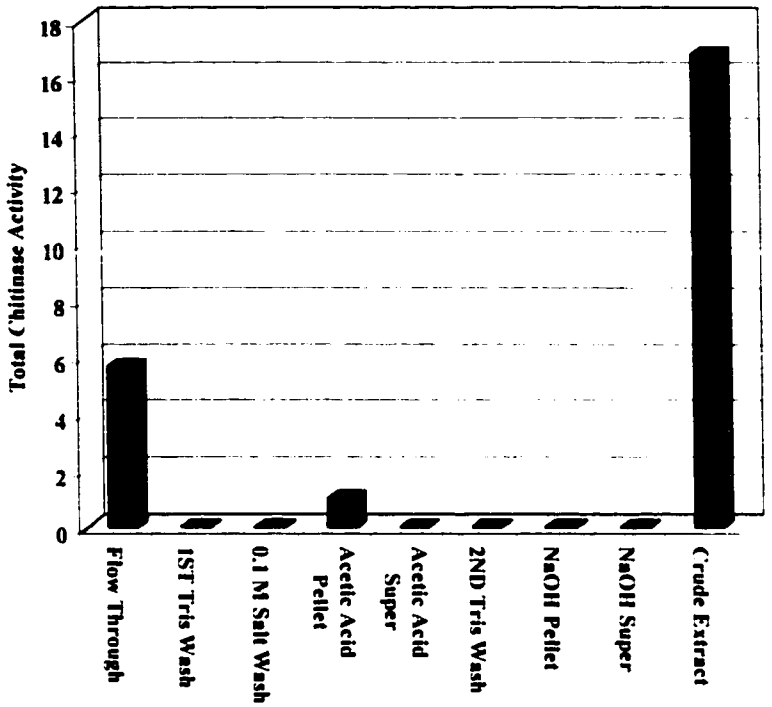


Figure IV-25: Total Chitinase Activity of Acid Chitin Column Fractions. Acid extract was fractionated on a chitin affinity column. Each fraction was dialyzed against 10 mM Tris-HCl, pH 8.0. A (100 µl) sample from each fraction was used to measure chitinase activity using the standard chitinase colorimetric assay.

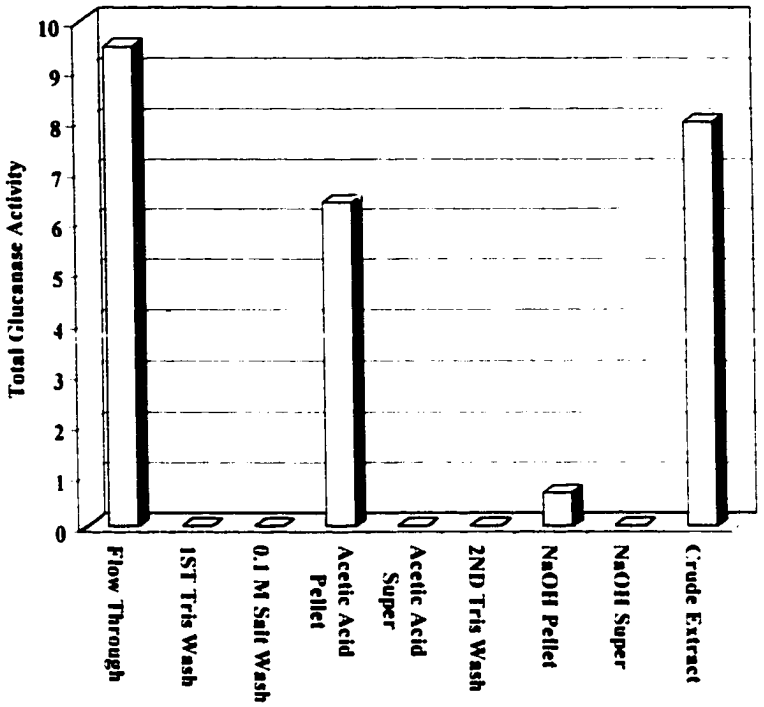


Figure IV-26: Total Glucanase Activity of Acid Chitin Column Fractions. *S. simplex* acid extract was fractionated on a chitin affinity column. Each fraction was dialyzed against 10 mM Tris, pH 8.0. A sample (100 µl) from each fraction was used to measure glucanase activity. The latter was determined using the standard glucanase colorimetric assay.

The SDS-PAGE protein banding profile of the fractions from the acid chitin column were very similar to, but not identical to, the SDS gel profile of neutral chitin column fractions (Figure IV-27). The lane with the acetic acid eluate contained much higher amounts of the 22 to 25-kDa bands as well as a number of lower molecular weight species. The sodium hydroxide eluate contained one major band around 32 kDa and prominent bands with molecular weights between 12 kDa and 24 kDa.

Other methods

Anion-exchange chromatography: Ion-exchange column chromatography was evaluated as a preliminary purification technique. Dialyzed neutral extract was fractionated on a Pharmacia Resource Q (RSQ) column. A total volume of 0.5 ml was loaded onto the column and the bound protein was eluted with a gradient from 0 to 1.0 M NaCl. Antifungal activity was detected in every RSQ fraction. There are at least two possible explanations for these results. First, there were multiple activities and/or multiple forms of the same activity; Alternatively proteins and/or other components in the extract interacted with the column and/or each other (data not shown).

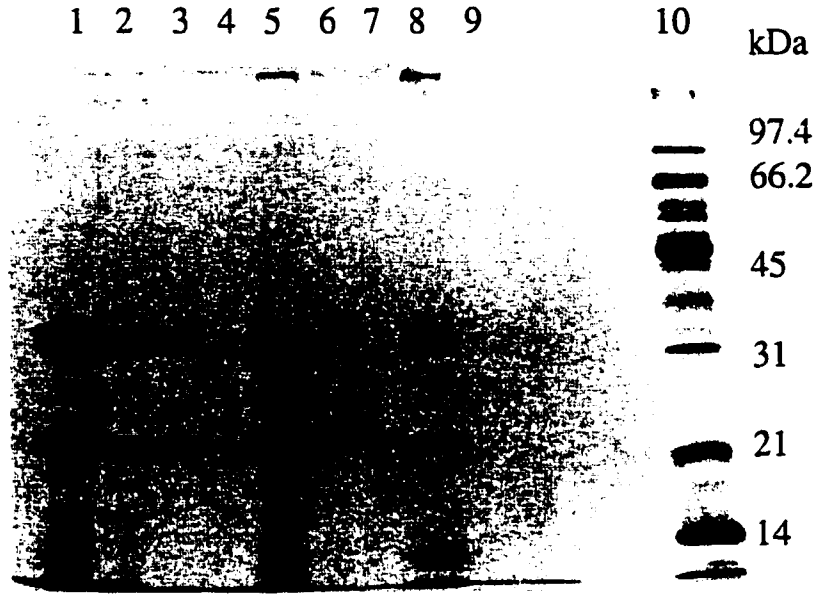


Figure IV-27: SDS-PAGE of Acid Chitin Column Fractions. Two hundred µl of each fraction was dried *in vacuo*, resuspended in 10 µl of sample buffer and boiled for 5 min before loading onto the gel. Lane 1, acid extract (100 µl dried); lane 2, flow through; lane 3, 0.1 M salt wash; lane 4, 1 M salt wash; lane 5, acetic acid eluate; lane 6, acetic acid supernatant; lane 7, second 1 M salt wash; lane 8, sodium hydroxide eluate; lane 9, sodium hydroxide supernatant; lane 10, molecular weight markers.

Either of these possibilities could account for the lack of separation between individual activities or components.

None of the fractions agglutinated rabbit red blood cells even though the starting material (neutral extract) exhibited very strong agglutination activity. The latter findings suggest that the agglutinating activity in the crude extract was highly charged and remained tightly bound to the RSQ column and was not eluted from the column under the conditions used herein. SDS-PAGE analysis of the RSQ fractions (data not shown) revealed several protein bands between 21 and 45 kDa in all the fractions. Thus, it was not possible to separate the antifungal activity into individual protein bands by anion-exchange chromatography using crude extracts. Therefore, ion-exchange column chromatography was not used as a method for preliminary fractionation.

Preparative isoelectric focusing: Dialyzed neutral extract was fractionated using a BioRad Rotofor isoelectric focusing chamber. Results of the antifungal bioassay of individual Rotofor fractions is presented in Figure IV-28. Fractions 2 to 10 and fractions 17, 18 and 20 exhibited slight inhibition of *A. flavus* hyphal growth. The recovery of total antifungal activity in the

Rotofor fractions was very low. The antifungal activity that survived IEF was mostly acidic in nature. Likewise, agglutinating activity did not survive Rotofor fractionation. The protein banding profile of Rotofor fractions was determined by SDS-PAGE. Fraction one to ten contained protein bands with estimated molecular weights between 21 and 50 kDa (data not shown). Overall, the antifungal activity was very low and

the gel profile indicated that there was very little separation of individual protein components. Therefore, preparative IEF was not used as a separation method.

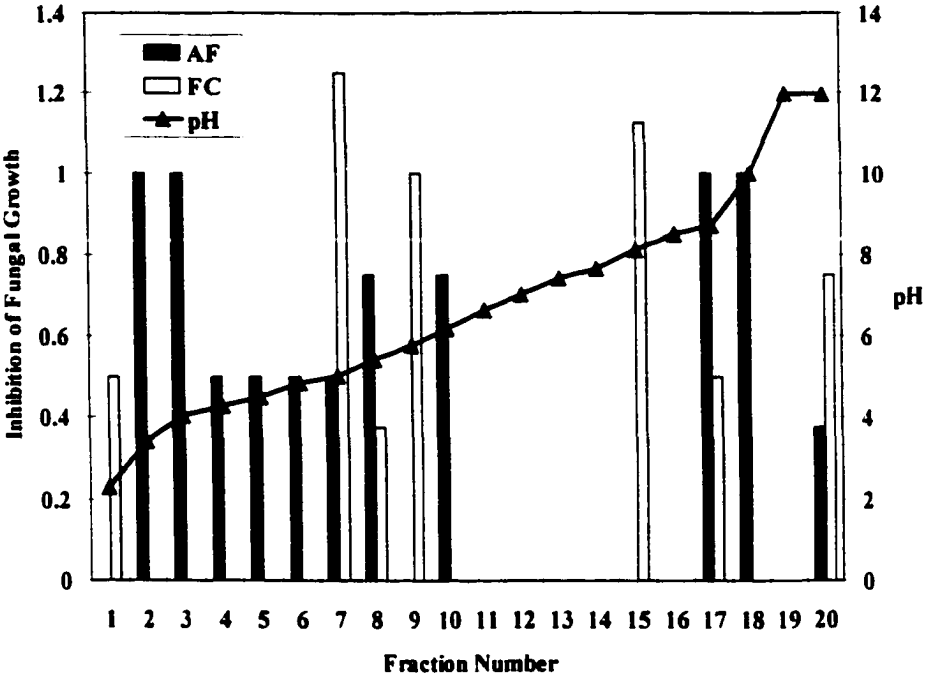


Figure IV-28: Antifungal Inhibition and pH Profile of *S. simplex* Neutral Extract Fractionated by Preparative Isoelectric Focusing. Twenty five ml of neutral extract was fractionated by preparative IEF. After fractionation, the pH of each sample was measured and fractions were dialyzed in salt followed by dialysis against 10 mM Tris-HCl, pH 8.0. A sample (100 µl) of each fraction was assayed for antifungal activity using *A. flavus*.

CONCLUSIONS

Based on the results of these preliminary studies, a number of antifungal (defense-related) proteins and/or other factors are present in extracts of *Swartzia simplex* seeds. These factors appear to be proteins based on heat sensitivity and relative size. Chitinases, glucanases, lectins and/or chitin-binding proteins present in these extracts may account for the antifungal activity. The most effective techniques for preliminary purification of the antifungal activities were chitin-affinity chromatography and size-exclusion chromatography. Other techniques such as ion-exchange chromatography and preparative IEF were less effective at this stage of purification.

Preliminary results suggest that an agglutinin may be responsible for at least part of the antifungal activity of these extracts. This conclusion is based on 1) the co-purification of agglutinating activity and antifungal activity by size-exclusion chromatography; 2) the concurrent loss of both activities after separation of acid extracts on the same column; 3) presence of both activities in chitin column fractions. Confirmation of this hypothesis will require further purification and characterization.

In the next chapter, the information gained herein will be applied to the development of a purification scheme for the isolation of antifungal proteins from the extracts of *S. simplex* seeds.

Chapter V : Purification of Antifungal Proteins from Seeds of *Swartzia simplex*

INTRODUCTION

In the preliminary studies, it was clear that substantial purification and separation of individual activities was achieved using either chitin-affinity chromatography or size-exclusion chromatography. The results of fractionation based on the charge of the protein (e. g. anion-exchange chromatography and preparative IEF) were less effective when working with relatively crude materials.

Preparative IEF can be an excellent bulk purification technique. With these particular samples, however, the recovery of activity after preparative IEF was very low. The low recovery of activity may be due to loss of activity at extremes of pH or decreased solubility and precipitation of proteins at or near their pI. Indeed, there was substantial precipitation during isoelectric focusing. Although this technique separated distinct

groups of antifungal activities with pI values in the acid, neutral and basic range, the poor recovery of activity precluded further use of this technique for the isolation of antifungal activities from *S. simplex*.

The potential separation possible with ion-exchange chromatography made this a good choice for fractionating proteins after initial separation by either chitin-affinity or size-exclusion chromatography. For further purification of antifungal activities from extracts of *S. simplex* seeds, anion-exchange chromatography was combined with either chitin-affinity chromatography or size-exclusion chromatography.

The specific components isolated may vary depending on the protocol used for the purification. To reduce the possibility that specific activities may be missed or lost with certain protocols, antifungal activities were isolated from seed extracts using several different protocols. Based on results presented in Chapter IV, four different schemes (Figure V-1) were developed for the purification of a broad range of antifungal activities. The four schemes will be referred to in subsequent sections of this chapter.

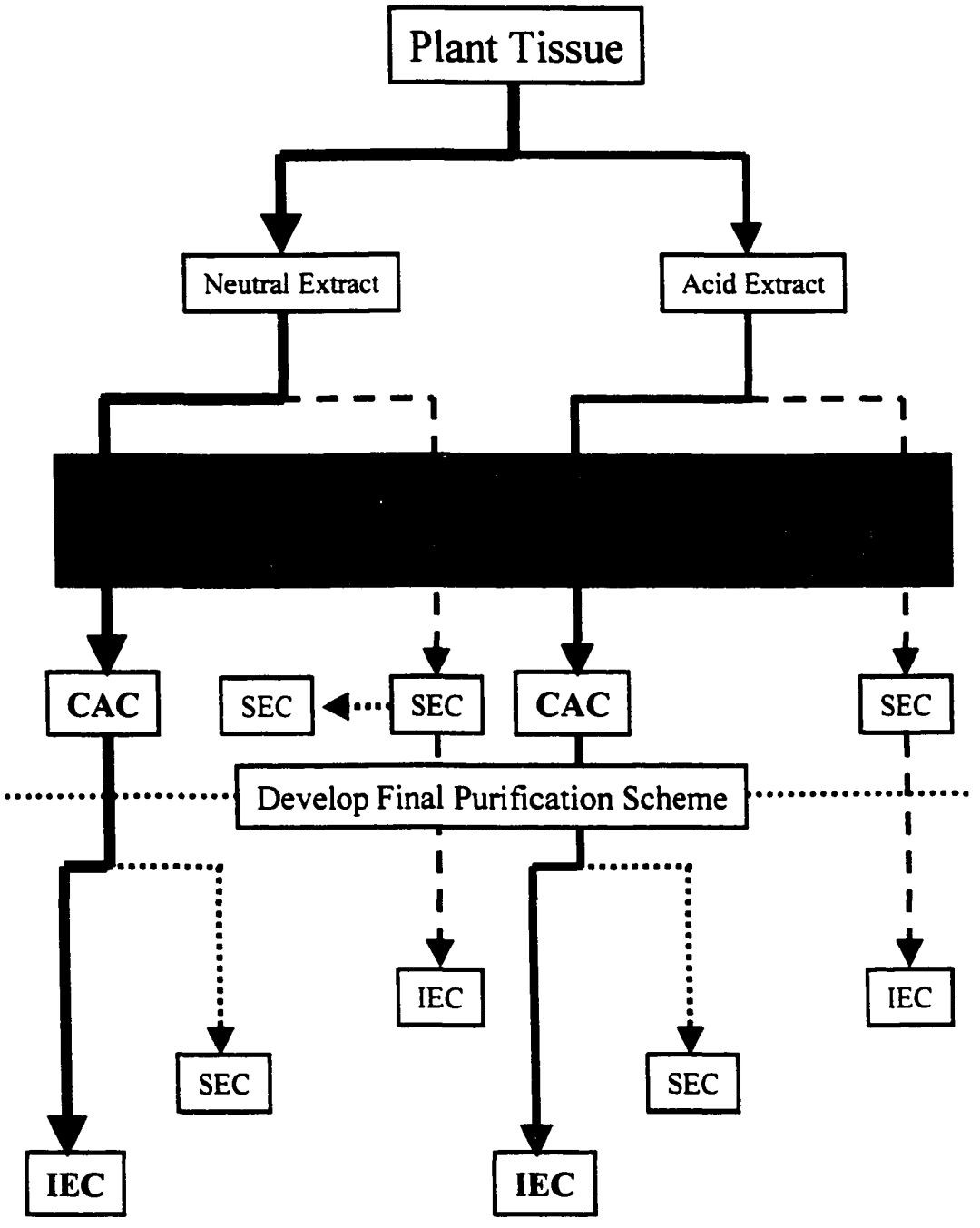


Figure V-1: Process for the Development of a Protocol for the Purification of Antifungal Proteins from Tropical Plants. SEC, size exclusion chromatography; IEC, ion exchange chromatography; IEF, isoelectric focusing; CAC, chitin affinity chromatography. Bold lines represent primary method used for the isolation of antifungal proteins. Dashed and dotted line represent alternative protocols for purification.

MATERIALS AND METHODS

The methods used within this chapter are the same as those described in Chapter IV unless otherwise noted. Please see the previous chapter for detailed information concerning methodology.

In several instances, Sephadex G-50 superfine resin was used for size based separation. The dry resin was hydrated in equilibrating buffer overnight, poured into a 1.6-cm x 100-cm column connected to the Pharmacia FPLC system and packed according to the manufacturer's instructions. The column was equilibrated with 10 mM Tris-HCl, pH 8.0, at a flow rate of 0.5 ml/min. Active fractions after Superose 12 chromatography were pooled and concentrated by ultrafiltration with a Centricon 10 concentrator. The concentrated fractions were centrifuged briefly to remove any insoluble material and 0.5 ml of the sample was loaded onto the G-50 column.

DEVELOPMENT OF FINAL PURIFICATION PROTOCOLS FOR THE ISOLATION OF ANTIFUNGAL ACTIVITIES FROM EXTRACTS OF *S. SIMPLEX* SEEDS

A combination of size-exclusion chromatography followed by anion-exchange chromatography was an effective means for purifying antifungal activity, agglutinin, chitinase and glucanase. This protocol was limited, however, by the amount of sample that can be loaded onto the gel filtration column and the dilution that occurs during separation. The latter is less important when techniques such as ion-exchange or affinity-chromatography follow the gel-filtration step. Chitin-affinity chromatography was not the best choice to follow size-based separations because both methods suffer from lack of resolving power. Both are more suitable as preliminary (bulk) fractionation methods.

Gel filtration separates proteins roughly based on size but this technique does not resolve proteins of very similar size. For example, gel filtration on Superose 12 would not separate a 20-kDa protein from a 30 to 40-kDa protein. Ionic, hydrophobic and affinity interactions between the sample and the column matrix and between individual components in the sample complicate these separations. For instance, some proteins remained bound to the Superose 12 column even after extensive washing of the

column with elution buffer. These materials were partially eluted with the addition of 1-2 M NaCl to the elution buffer and only completely removed by washing the column with 1 M NaOH. Under these conditions, activity may be lost.

As another example, several of the antifungal activities were eluted as if they were less than 10 kDa in size, yet the analysis of SDS profiles suggested that the proteins were greater than 20-30 kDa. Obviously, these proteins were interacting with the column matrix. Chitinases and glucanases, on the other hand, were eluted as much larger species. These proteins may be aggregating or interacting with other components adding to their apparent mass. These subtle features of column chromatography can be used to advantage in achieving unique separations.

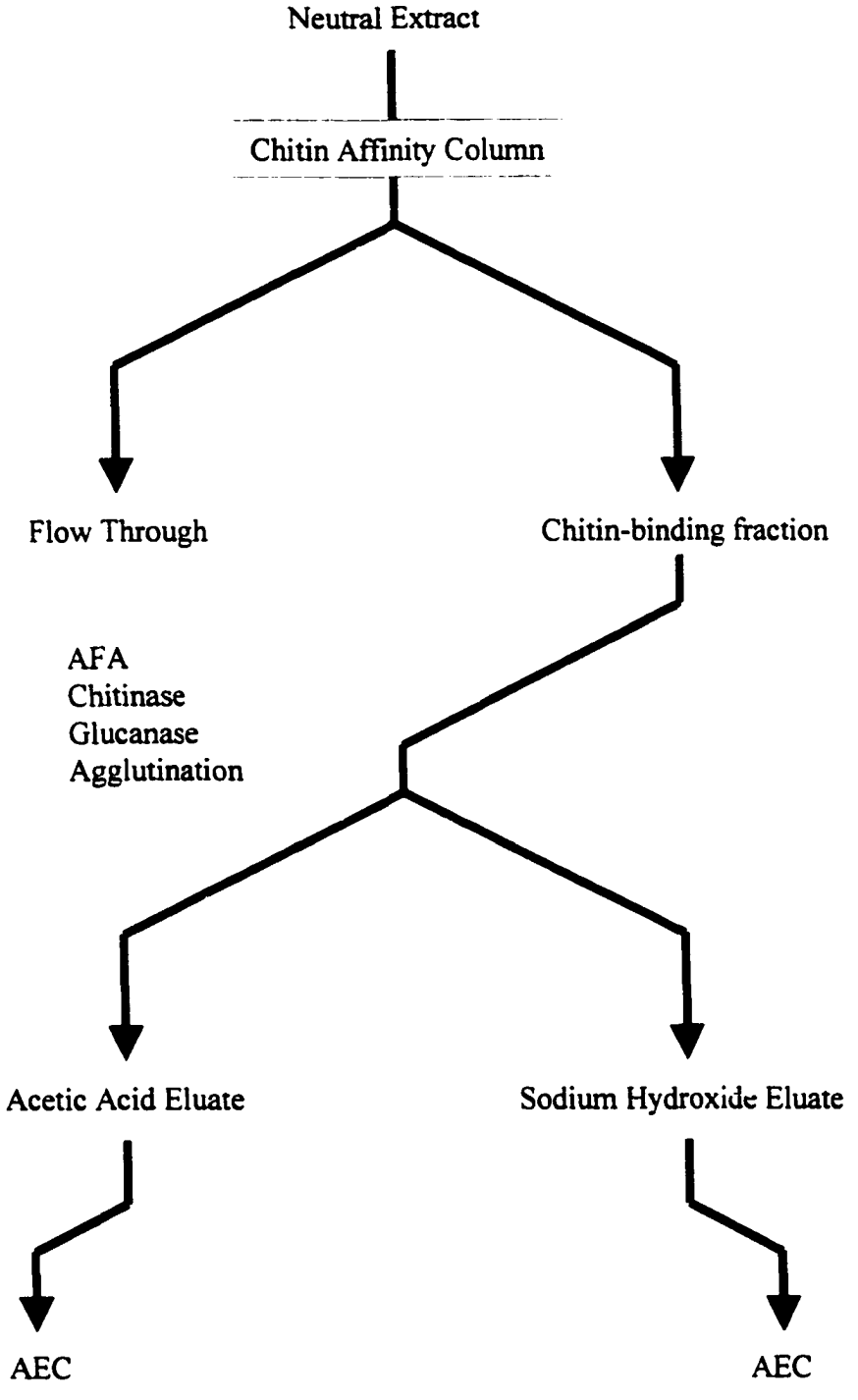
Chitin-affinity chromatography suffers from the disadvantage that there are many chitin-binding activities in these extracts. Not all of the materials bound to the column interact specifically with chitin. Chitin is highly charged and many proteins may bind through ionic and not affinity interactions. Other proteins may bind to sugar residues and not to chitin *per se*. Although gel filtration was used to further purify specific proteins from the chitin-affinity column, the results were not very satisfactory (Data not

shown). Gel filtration fractions were not subjected to chromatography on chitin for the very same reasons.

Ion-exchange chromatography on high performance resins such as Pharmacia Resource-Q (RSQ) can be an extremely effective means for protein purification. As shown earlier, these high performance techniques did not provide satisfactory separations of crude extracts of *Swartzia* seeds. This lack of separation may be specific to extracts of this species. The extract contains a complex mixture of closely related proteins. This technique is much more effective when used after an initial purification step. As just stated, gel filtration followed by anion-exchange chromatography was effective in providing highly purified proteins suitable for further analysis and characterization but was limited in terms of the amounts of protein that can be handled efficiently. For this reason, the primary purification scheme (Scheme I and III, Figure V-1) used in subsequent experiments combined chitin-affinity chromatography with anion-exchange chromatography.

Antifungal activities present in neutral and acid extracts of *S. simplex* seeds were purified using the protocols outlined in Scheme I and III (Figure V-1), respectively. The detailed protocol for the purification of antifungal activities and chitinases from neutral extract is outlined in Figure V-2.

Figure V-2: Purification of Antifungal Activities by Chitin-affinity Chromatography



PURIFICATION OF ANTIFUNGAL ACTIVITIES BY CHITIN-AFFINITY CHROMATOGRAPHY

Further purification of active fractions from the neutral chitin column

Flow through: The flow through obtained after chitin-affinity chromatography of the neutral extract completely inhibited *A. flavus* conidial germination. In addition, this fraction contained chitinase, glucanase and agglutinating activity. These proteins may not bind to the chitin-affinity column for several reasons. Either the proteins had no affinity for chitin or the column was overloaded. Further purification of these activities from the flow through was not carried out. Emphasis was placed on the chitin-binding fractions from this column. Further work is required to isolate the specific activities present in the flow through.

Acetic acid eluate: The resuspended ammonium sulfate pellet from the acetic acid eluate of the neutral chitin column completely inhibited conidial germination of *A. flavus*. Chitinases and glucanases were also detected in this fraction. Although the same general activities (i. e. chitinase,

agglutination and antifungal activities) were present in the flow through from the neutral chitin column, the SDS gel profile revealed that, in general, there were fewer protein bands in the acetic acid eluate and the common bands were enriched. Therefore, the acetic acid eluate was chosen over the flow through for further purification of chitinase, glucanase and antifungal activities.

Ion-exchange chromatography of acetic acid eluate: The acetic acid eluate was precipitated with ammonium sulfate and the resuspended pellet was subjected to anion-exchange chromatography on a Pharmacia RSQ column. The column was equilibrated with 10 mM Tris-HCl, pH 8.0. Five hundred µl of the resuspended and dialyzed acetic acid pellet was loaded onto the column and bound proteins were eluted with a linear gradient from 0 to 1 M NaCl. A very good separation was obtained based on the absorbance profile (Figure V-3) and the banding pattern after SDS-PAGE (Figure V-4).

Fractions were dialyzed extensively against 10 mM Tris-HCl, pH 8.0, to remove salt before testing for antifungal and chitinase activity. Complete inhibition of *A. flavus* conidial germination was observed in fractions 3, 5-7, 9, 10, 13 and 14 (Figure V-5). Fractions 1, 2, 8, 12 and 15 did not show any

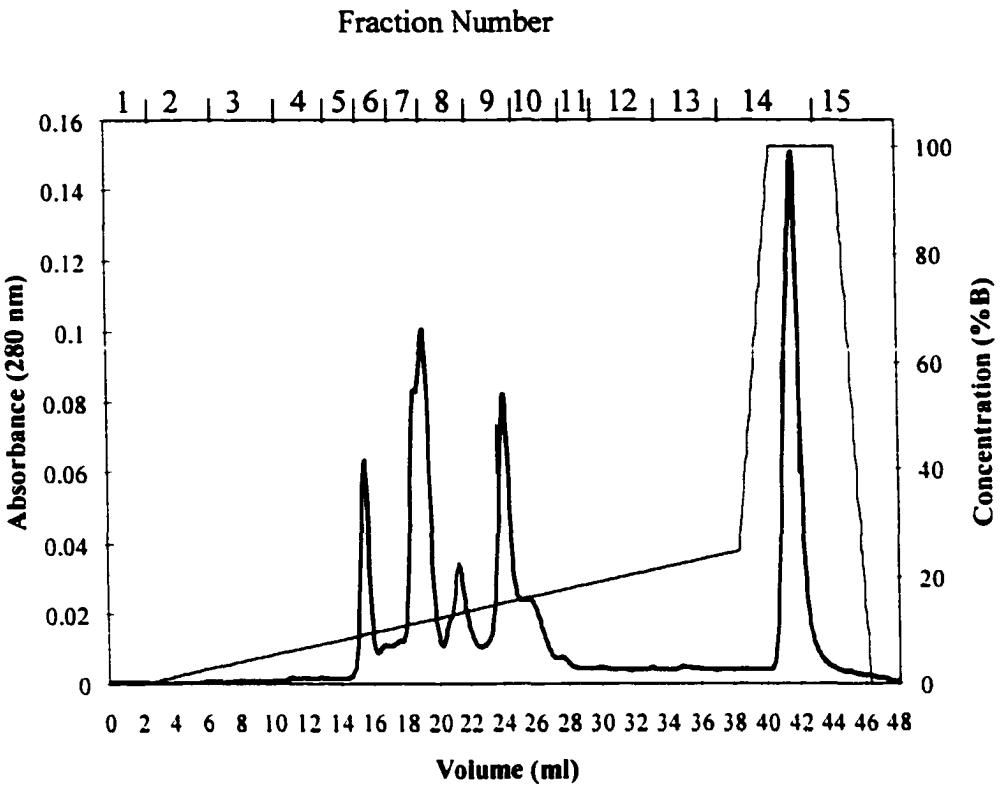


Figure V-3: Absorbance Profile of Anion Exchange Column Chromatography of Acetic Acid Eluate from a Neutral Chitin Column. Five hundred µl of acetic acid eluate was applied to the column equilibrated in 10 mM Tris-HCl, pH 8.0. Bound proteins were eluted with a linear gradient of sodium chloride in the same sample buffer. Fractions were collected manually based on the absorbance at 280 nm. Position of the fractions is noted on the top of the figure.

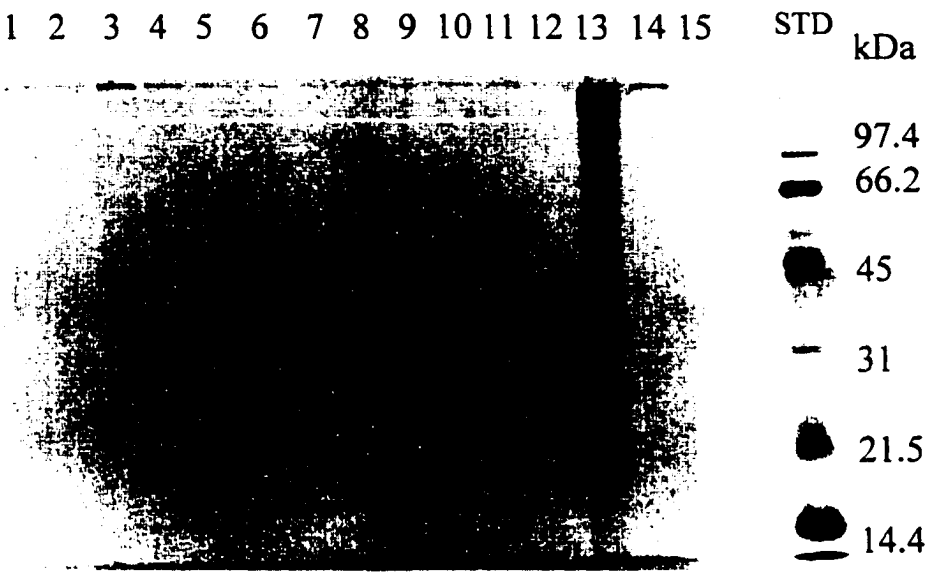


Figure V-4: SDS-PAGE Analysis of Fractions Obtained after Anion Exchange Chromatography of the Acetic Acid Eluate from the Neutral Extract. A sample (200 µl) from each fraction was dried *in vacuo*, resuspended in 10 µl of sample buffer and boiled for 5 min before loading onto the gel. Lane 1 to 12, RSQ fractions (see Figure V-3) 1 to 12; lane 13, fraction 14; lane 14, fraction 15; lane 15, fraction 13. The gel was silver stained to reveal protein bands. STD= molecular weight standards.

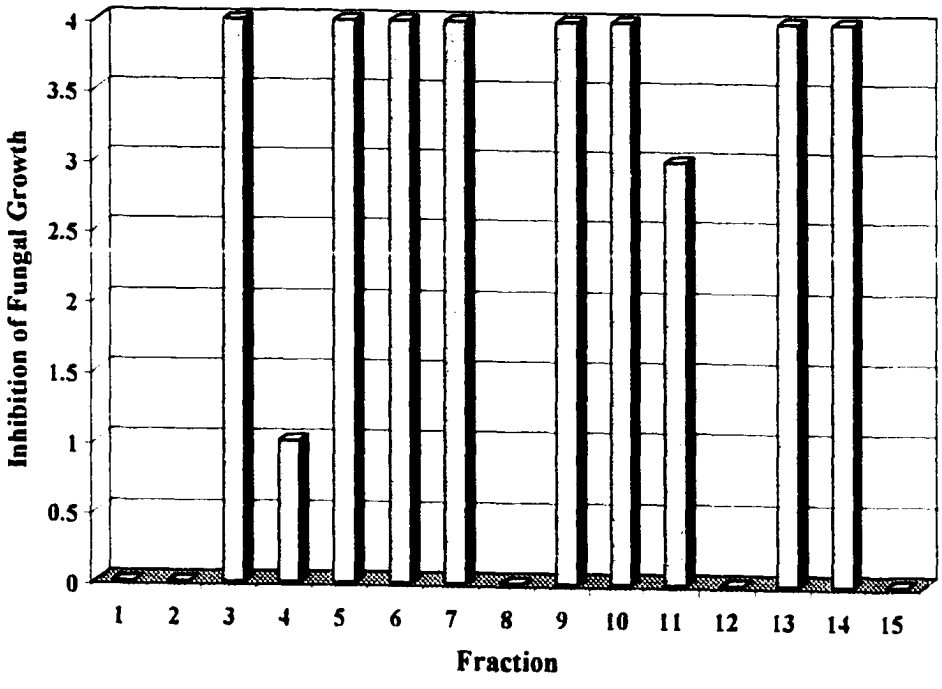


Figure V-5: Antifungal Activity of Fractions Obtained After Anion Exchange Chromatography of the Acetic Acid Eluate from a Neutral Chitin Column. Acetic acid eluate was fractionated on a Pharmacia RSQ column. All fractions were dialyzed against 10 mM Tris-HCl, pH 8.0, to remove salt. A sample from each dialyzed fraction was concentrated (2x) and 100 µl of the concentrated sample was assayed for antifungal activity against *A. flavus*.

antifungal activity. Chitinases were detected in fractions 5-10 (Figure V-6). Several protein bands of approximately 30-34 kDa were observed in SDS gels of all six fractions. Fraction 5 contained one major 30-kDa-protein band, indicating that the sample was nearly homogeneous. Fractions 6, 8 and 9 each had two protein bands (approximately 31-34 kDa). There was one major band and several minor bands in fraction 7 and three minor bands in fraction 10. The multiplicity of protein bands may reflect different forms of the same activity including post-translational modifications. Alternatively, a protein may be composed of non-identical subunits. Although this is less likely in the case of chitinases (chitinases are generally monomeric proteins), the possibility certainly exists for other activities. The presence of multiple bands does not infer that the protein was impure. Fraction 10 contained three bands and fraction 7 contained four bands.

Fraction 7 was further purified by micro-HPLC at the OUHSC (data not shown). SDS gel profile (see Chapter VI) of the micro HPLC fractions revealed one 32-kDa band in the peak fraction. A second band of similar size was evident in the trailing edge of the peak fraction. Purified proteins in these RSQ fractions were further characterized (see Chapter VI).

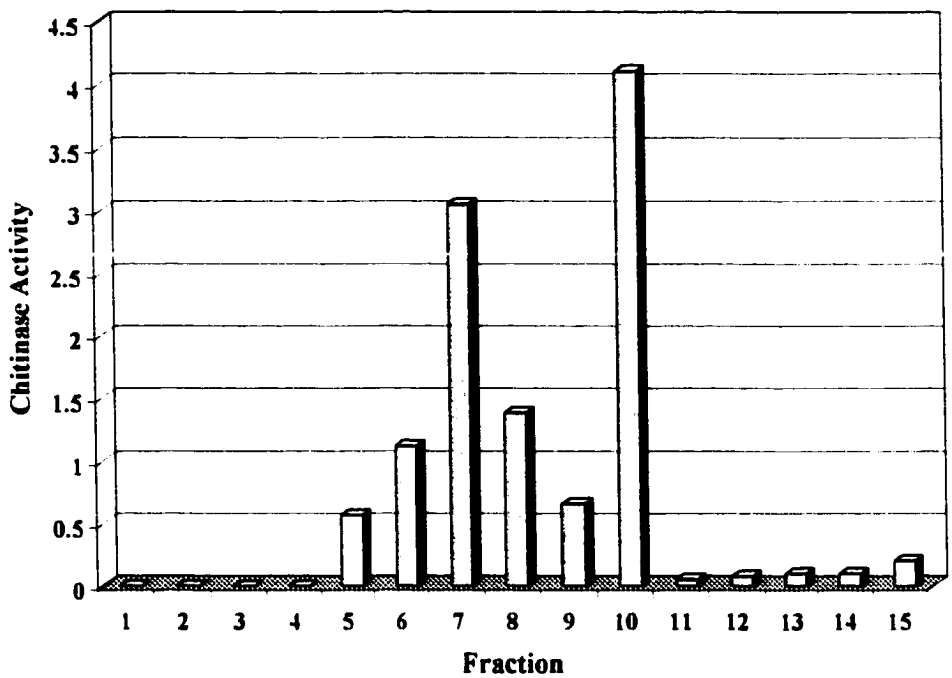


Figure V-6 : Total Chitinase Activity of Anion Exchange Fractions of the Acetic Acid Eluate from a Neutral Chitin Column. Acetic acid eluate was fractionated on an anion exchange column. Each fraction was dialyzed against 10 mM Tris-HCl, pH 8.0, and a sample from each fraction was concentrated (2x). One hundred µl of the concentrated sample from each fraction was evaluated for chitinase activity using the colorimetric chitinase assay.

Sodium hydroxide eluate: The sodium hydroxide eluate from the neutral chitin column was concentrated with ammonium sulfate. The pellet was resuspended and dialyzed extensively. The sodium hydroxide eluate exhibited antifungal activity as well as chitinase and glucanase activity. Several bands were detected after SDS-PAGE. To further purify these activities, the sodium hydroxide eluate was fractionated by anion-exchange chromatography.

Anion-exchange chromatography of sodium hydroxide eluate: The sodium hydroxide eluate was fractionated by anion-exchange chromatography on a Pharmacia RSQ column. The absorbance profile is presented in Figure V-7. Eleven fractions were collected and assayed for antifungal activity. Moderate to strong antifungal activity was present in all the fractions except 2, 6 and 8 (Figure V-8). Among the active fractions, the best activity was in fractions 4 and 5. Both of these fractions strongly inhibited *A. flavus* hyphal growth and conidial germination. Strong inhibition was also observed in fractions 1 and 10. This activity inhibited hyphal growth but not conidial germination. Moderate inhibition of *A. flavus* hyphal growth was observed in wells containing fractions 3, 7, 9 and 11.

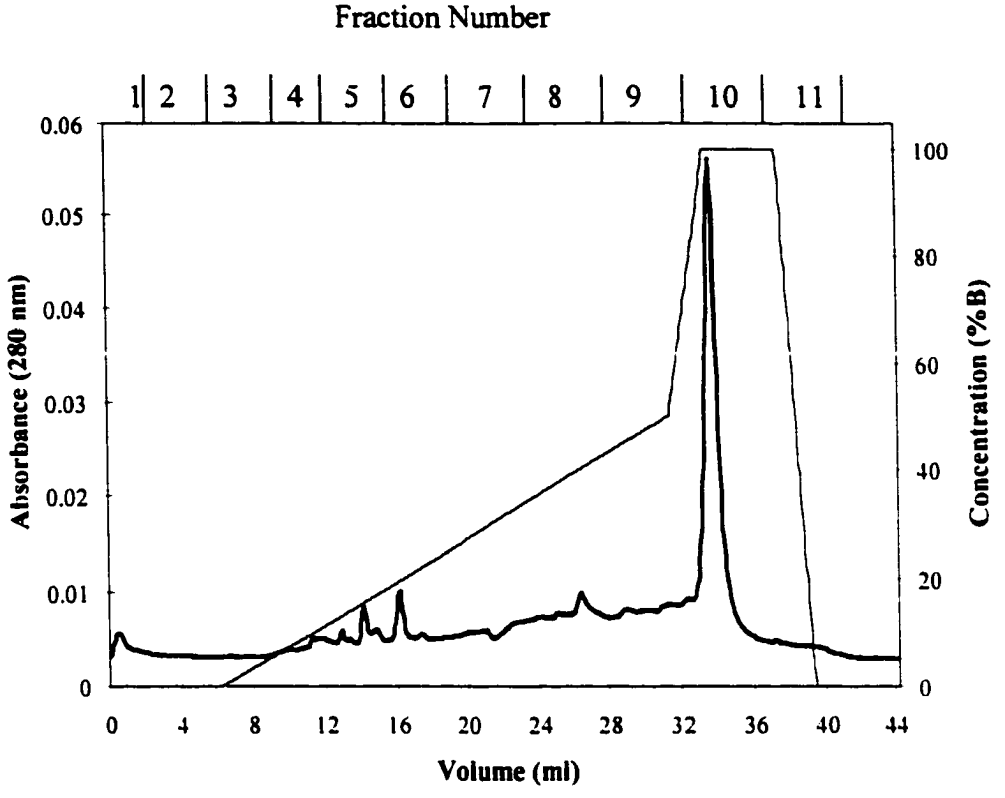


Figure V-7 : Absorbance Profile of Anion Exchange Column Chromatography of the Sodium Hydroxide Eluate from the Neutral Chitin Column. Five hundred µl of sodium hydroxide eluate was applied to the column and bound proteins were eluted with a linear gradient of sodium chloride in 10 mM Tris-HCl, pH 8.0. Fractions were collected manually based on the absorbance at 280 nm. The specific fractions were noted on the top of the figure.

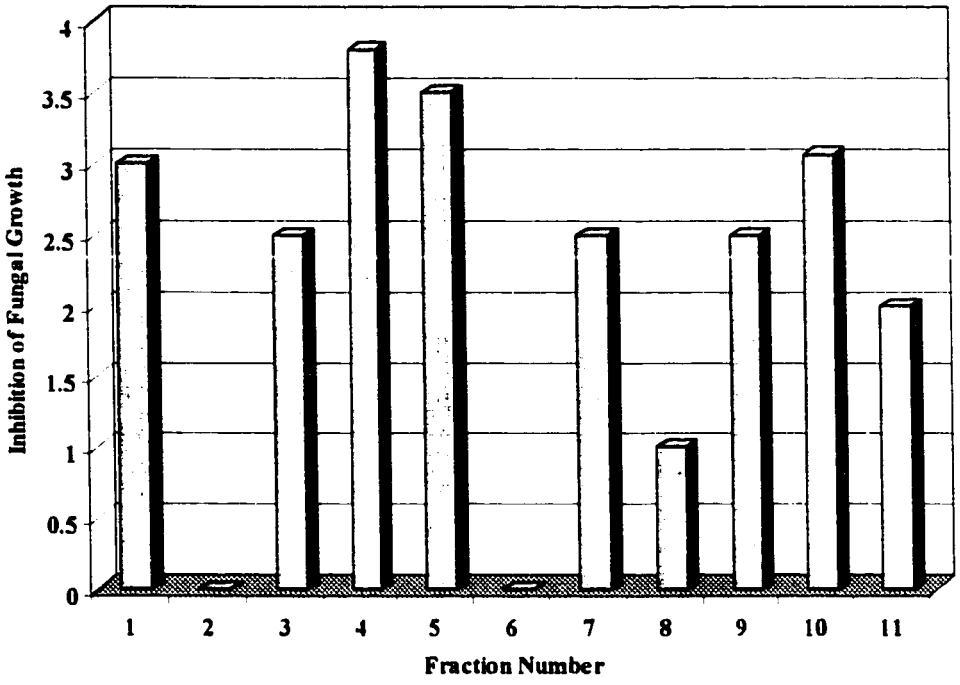


Figure V-8: Evaluation of the Antifungal Activity of Fractions after Anion Exchange Chromatography of the Sodium Hydroxide Eluate from a Neutral Chitin Column. Sodium hydroxide eluate from the neutral chitin column was fractionated on a Pharmacia RSQ column. All the fractions were dialyzed against 10 mM Tris-HCl, pH 8.0, and assayed for antifungal activity against *A. flavus*.

Prolonged incubation of the assay revealed the stable nature and durability of the activity in fractions 9-10. These two fractions exhibited strong inhibition of hyphal growth even after 4 weeks. Although fraction 11 caused moderate inhibition after 48 h, hyphal growth remained the same even after 4 weeks of incubation. The activity of the remaining active fractions (1, 3-5, 7-8) declined after 4 weeks of incubation and hyphal growth in the microtiter wells containing these fractions was comparable to the hyphal growth in the control wells. This may be due to the loss of activity. Alternatively, even though the rate of fungal growth may have been reduced dramatically, the cumulative, albeit slow, growth of the fungus may have filled the well after prolonged incubation. This would be difficult to distinguish such slow growth from the growth of the controls which filled the wells within 48 h. SDS gel profiles for these fractions are presented in Figure V-9.

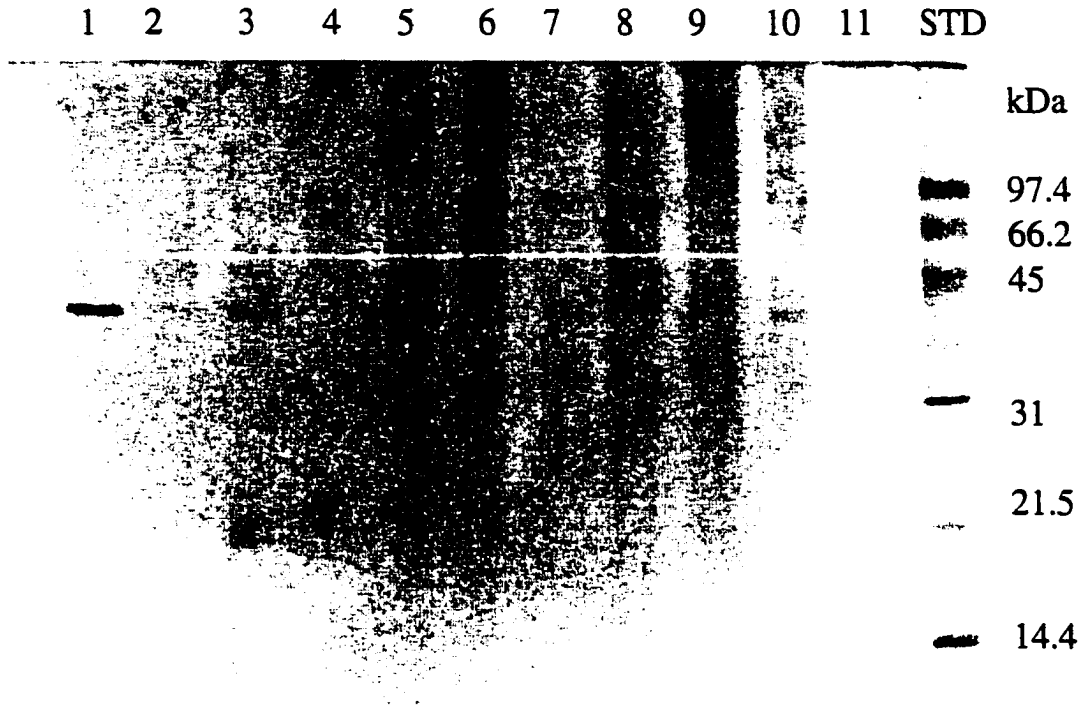


Figure V-9 : SDS-PAGE Gel of Fractions Obtained after Anion Exchange Chromatography of the Sodium Hydroxide Eluate from a Neutral Chitin Column. Sodium hydroxide eluate was fractionated on a Pharmacia RSQ column. A 200 µl sample from each fraction was dried *in vacuo*, resuspended in 10 µl of sample buffer and boiled for 5 min before loading onto the 12.5% SDS gel. Lane 1 to 11, RSQ fractions 1 to 11. The gel was silver stained to detect protein bands. STD = molecular weight standards.

Further fractionation of chitinase and glucanase-containing fractions from the acid chitin column

In general, chitinases purified by anion-exchange chromatography of the eluates from the acid chitin column were purer than chitinases isolated by the same procedures but with neutral extract. The amount of chitinase extracted, however, was much lower when the acid extract was used. A comparison of the results using the two different extraction procedures is presented in Table V-1. The total chitinase activity in the crude acid extract (AEx) was only one-third of the activity in the neutral extract (NEx). Overall, the specific activities in the crude acid extract and fractions from the acid chitin column were much lower than the corresponding fractions derived from the neutral extract. This result suggests that specific forms of chitinase with high unit activity may have been lost during acid extraction. It should be noted that the antifungal activity of these fractions was very good considering the low amount of protein present.

The acetic acid eluate from the acid chitin column was further fractionated by anion-exchange chromatography. The absorbance (280 nm) profile of the column (Figure V-10) showed 4 distinct peaks in fractions 3, 4 and 5 that were eluted at low concentrations of salt and one minor peak

Table V-1: Comparison of Extraction Methods for the Isolation of Chitinase from Extracts of *S. simplex* Seeds.

Fraction	Activity (μmol/h/ml)	Protein (mg/ml)	Volume (ml)	Specific Activity (μmol/h/mg)	Total Activity (μmol/h)	Yield (%)
NEUTRAL						
Crude	7.7	1.56	5	5.0	38.7	100
FT	5.7	0.24	5	23.9	28.7	74.2
ACP	5.0	0.42	1	12.0	20.0	51.7
SHP	0.11	0.34	0.75	0.34	0.86	2.2
ACID						
Crude	2.7	0.9	5	3.0	13.4	100
FT	0.9	0.8	5	1.1	4.5	33.6
ACP	0.4	0.3	2	1.4	0.8	8.5
SHP	0.008	0.28	1.3	0.028	0.01	>0.01

after high salt elution. Twenty fractions were collected and pooled based on the absorbance profile, dialyzed against 10 mM Tris-HCl, pH 8.0 and assayed for antifungal as well as chitinase activity. Chitinase activity (Figure V-11) was detected in the peak fractions (3, 4 and 5). The fungal bioassay revealed antifungal activity in fraction 4 and fraction 6 (Figure V-12). The SDS-PAGE profile (Figure V-13) revealed very distinct 31-32 kDa protein bands in the chitinase-containing fractions (3-5).

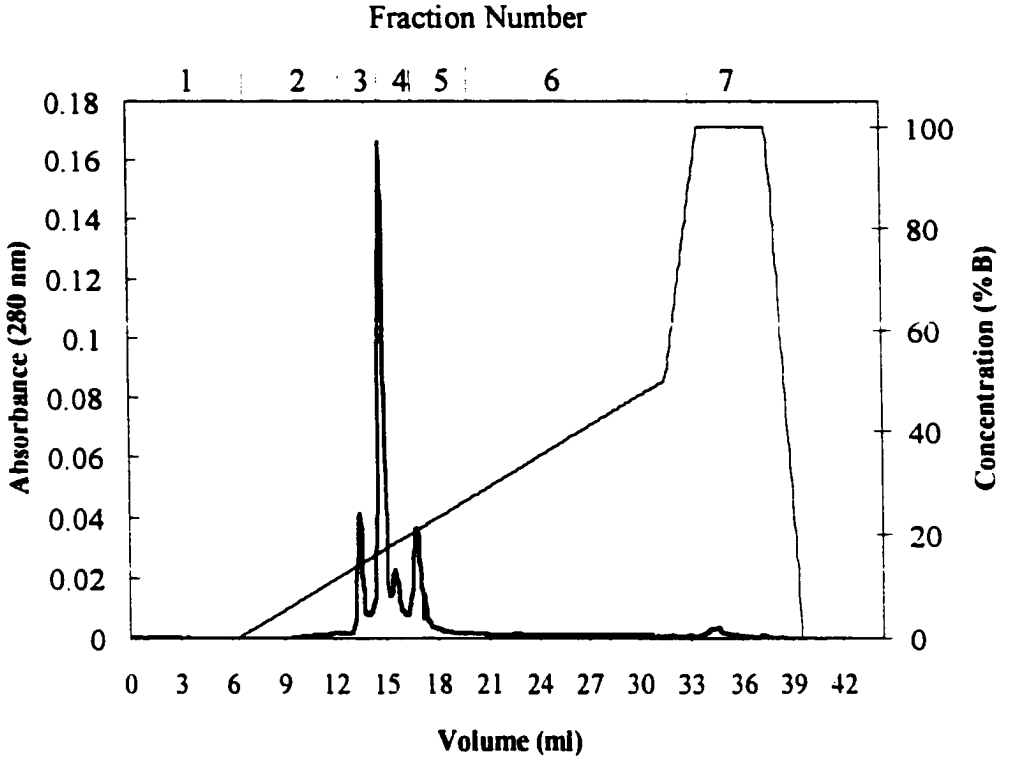


Figure V-10: Absorbance Profile of Anion Exchange Chromatography of the Acetic Acid Eluate from the Acid Chitin Column. Five hundred µl of the acetic acid eluate was applied to the RSQ column equilibrated with 10 mM Tris-HCl, pH 8.0. Bound proteins were eluted with a linear gradient of sodium chloride in the same buffer.

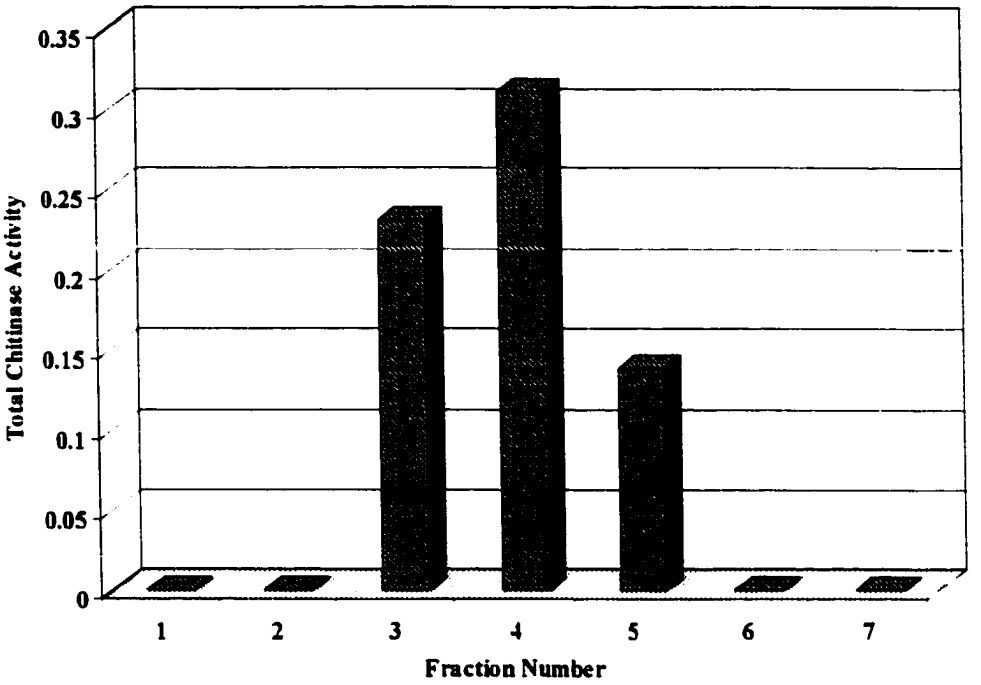


Figure V-11: Total Chitinase Activity of the Anion Exchange Fractions of the Acetic Acid Eluate from the Acid Chitin Column. Acetic acid eluate from the acid chitin column was fractionated on a Pharmacia RSQ column. Each fraction was dialyzed against 10 mM Tris-HCl, pH 8.0., and a sample from each fraction was concentrated (2x). One hundred µl of the concentrated sample from each fraction was evaluated using the colorimetric chitinase assay.

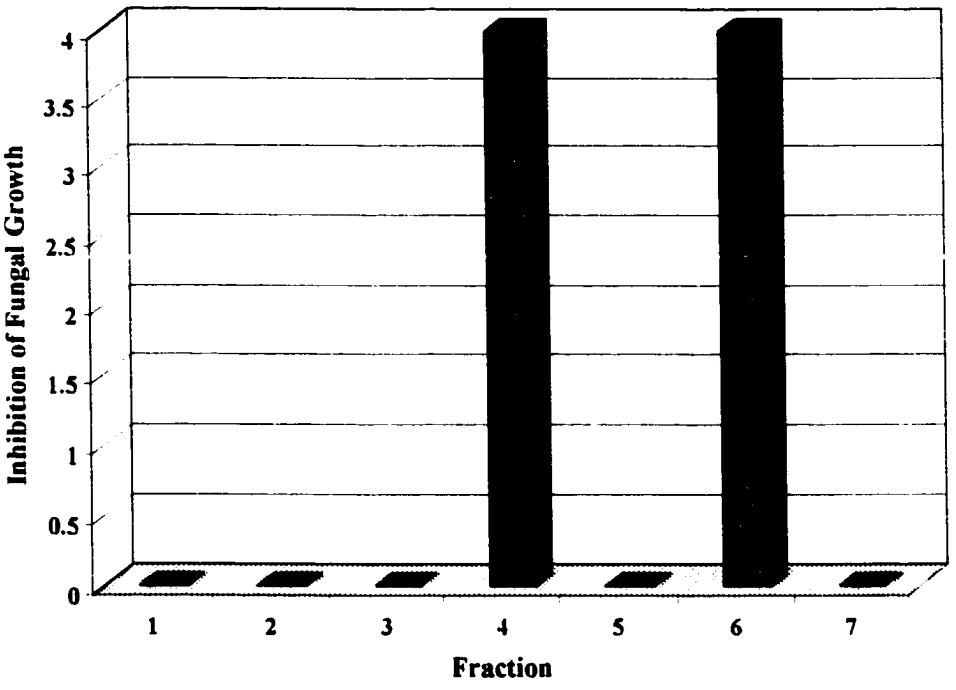


Figure V-12: Antifungal Activity of Fractions Obtained after Anion Exchange Chromatography of the Acetic Acid Eluate from the Acid Chitin Column. Fractions from the anion exchange separation were dialyzed against 10 mM Tris-HCl, pH 8.0, and concentrated (2x). One hundred µl of the concentrated sample from each fraction was assayed for antifungal activity against *A. flavus*.

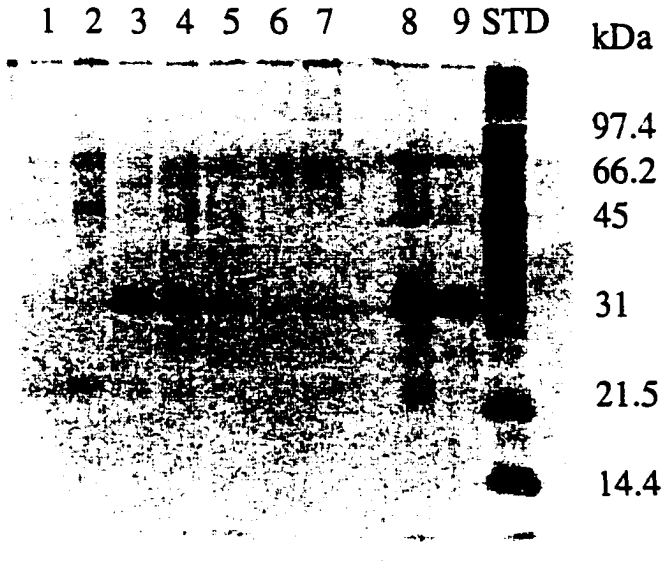


Figure V-13: SDS-PAGE Gel of Fractions Obtained after Anion Exchange Chromatography of the Acetic Acid Eluate from the Acid Chitin Column. The acetic acid eluate was fractionated on a Pharmacia RSQ column. Two hundred µl from each fraction was dried *in vacuo*, resuspended in 10 µl of sample buffer and boiled for 5 min before loading onto the 12.5% SDS gel. Lane 1 to 7, RSQ fractions 1 to 7; lane 8, neutral chitin column acetic acid eluate; lane 9, acid chitin column acetic acid eluate. STD = molecular weight standards.

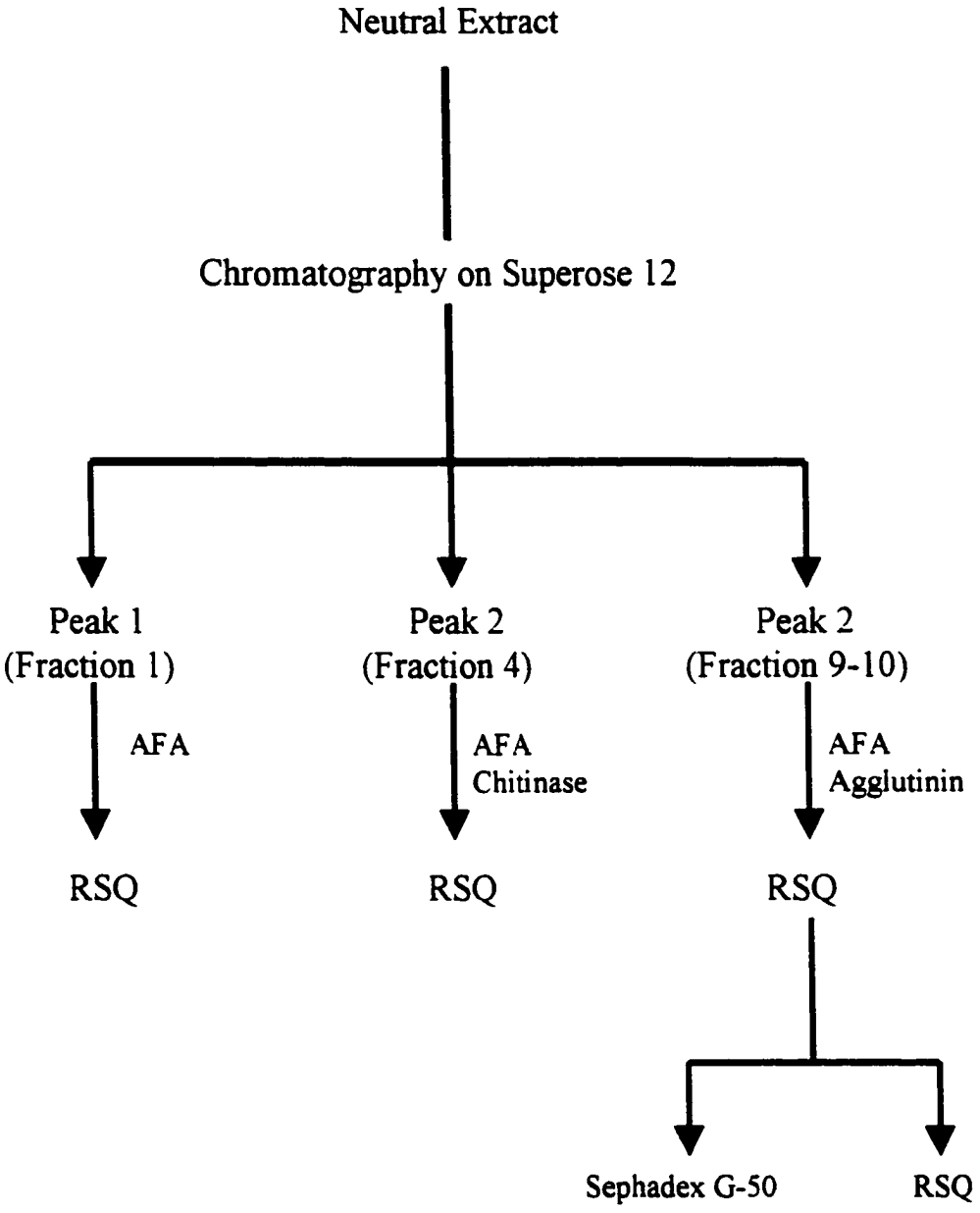
ALTERNATIVE PURIFICATION PROTOCOLS FOR THE ISOLATION OF ANTIFUNGAL PROTEINS FROM EXTRACTS OF *S. SIMPLEX* SEEDS

The primary method for the isolation of antifungal proteins, especially chitinases and other chitin-binding proteins involved the use of a combination of chitin-affinity chromatography followed by anion-exchange chromatography. Alternative protocols were developed for the purification of antifungal proteins from both neutral and acid extracts using size-exclusion chromatography and either anion-exchange chromatography or a second gel filtration step. The protocols for these separations are outlined in Figure V-1 Schemes II and IV. Although the latter methods were not the best methods for the isolation of chitinase and other chitin-binding proteins, these methods were very effective for the isolation of other classes of active proteins. To date, none of the other active proteins identified as part of these studies has been characterized. Results of these studies are included herein for future reference.

Purification of antifungal activity from Superose 12 fractions from neutral extracts of *S. simplex* seeds

After fractionation of neutral extract of *S. simplex* seeds by size-exclusion chromatography on Superose 12, three zones of antifungal activity were detected, fraction 1, fraction 4 and fraction 9-10. Fraction 4 also contained chitinase and glucanase activity and fractions 9 and 10 contained agglutinating activity. The three zones of antifungal activity were further purified by either anion-exchange chromatography or by size-exclusion chromatography on Sephadex G-50 as outlined in Scheme II (Figure V-1). A flow chart for these experiments including a summary of key results is presented in Figure V-14. Although Scheme II was not used as the primary method for the purification of antifungal activity from neutral extracts, some of the actual results of these studies are included herein for future reference. The techniques outlined in Scheme II may be useful for the purification of other antifungal activities identified in these studies but not yet purified and characterized.

Figure V-14: Alternative Protocol for the Isolation of Antifungal Proteins from Neutral Extracts of *S. simplex* Seeds.



Anion-exchange chromatography of Superose 12 fraction 1: Fraction 1 (10 ml) was loaded onto the RSQ column and bound proteins were eluted with a linear gradient of NaCl in 10 mM Tris-HCl (pH 8.0). Fractions were collected and pooled based on the absorbance at 280 nm. Pooled fractions were dialyzed against 10 mM Tris-HCl, pH 8.0, and assayed for antifungal activity. Antifungal activity (data not shown) was present in all the fractions except fractions 1 to 4 (pooled fraction 1). Samples from each fraction were subjected to SDS-PAGE analysis. A protein band with an estimated molecular weight between 38 and 41 kDa along with three to four bands in the range of 50-60 kDa were detected in the active fractions after silver staining the gel (data not shown).

Anion-exchange chromatography of Superose 12 fractions containing chitinase and glucanase: Although antifungal activity was only detected in fraction 4, fractions 4 and 5 containing chitinase and glucanase were pooled and 2 ml of the pooled material was applied to the RSQ anion-exchange column in order to purify the antifungal activity. The absorbance and gradient profiles for the RSQ separation of the chitinase/glucanase-containing fractions are presented in Figure V-15.

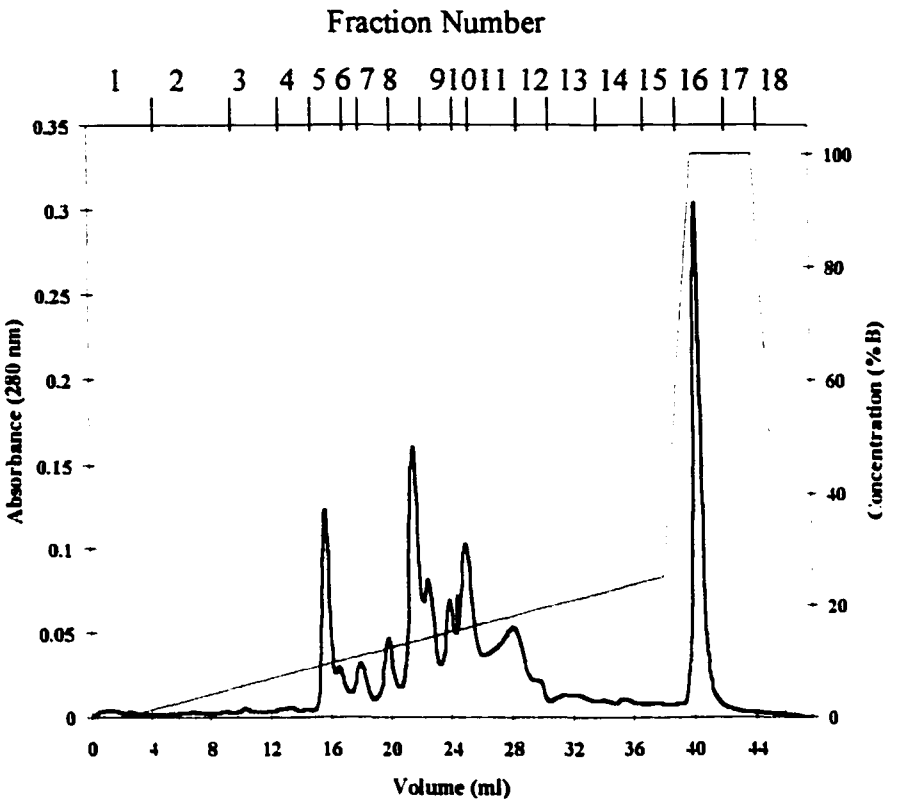


Figure V-15: Absorbance Profile of Fractions Obtained after Anion Exchange Chromatography of Neutral Extract Superose 12 Fractions 4 and 5. After chromatography of neutral extract on Superose 12, fraction 4 and 5 containing antifungal activity, chitinase and glucanase were pooled and 500 µl of the pooled fraction was injected onto a RSQ anion exchange column. The column was equilibrated with 10 mM Tris-HCl, pH, 8.0 and the bound proteins were eluted with a linear gradient from 0-1 M NaCl in the same buffer. Fractions were collected manually and pooled based on the absorbance profile.

Twenty fractions were collected from the RSQ column and these fractions were dialyzed and then assayed for antifungal activity. Antifungal activity was observed in fraction 3, 5-7, 9 and 12 to 17 (Figure V-16).

Ten distinct peaks were visible in the absorbance profile. Chitinases were detected in 6 of these peaks based on results from the colorimetric chitinase assay (Figure V-17). The eighth peak (fraction 17) did not exhibit any chitinase activity and the SDS gel profile (Figure V-18) did not show any visible protein band in this fraction.

Anion-exchange chromatography of Superose 12 fractions 9 and 10: Fraction 9 and 10 from the neutral extract Superose 12 column were pooled and 10 ml of the pooled fraction was subjected to anion-exchange chromatography as stated above. Fractions were collected, dialyzed separately and assayed for antifungal activity. Activity was present in all 24 fractions. The protein-banding pattern was determined by SDS-PAGE. The pattern in each fraction was similar with proteins of approximately 28 kDa and less than 21 kDa present in all the fractions. The major difference in the fractions was the presence of a major band of about 38 to 41 kDa in fractions 1-5 and 13-17. The lower molecular weight bands present in all the fractions appear to correlate with the antifungal activity. Note that the age

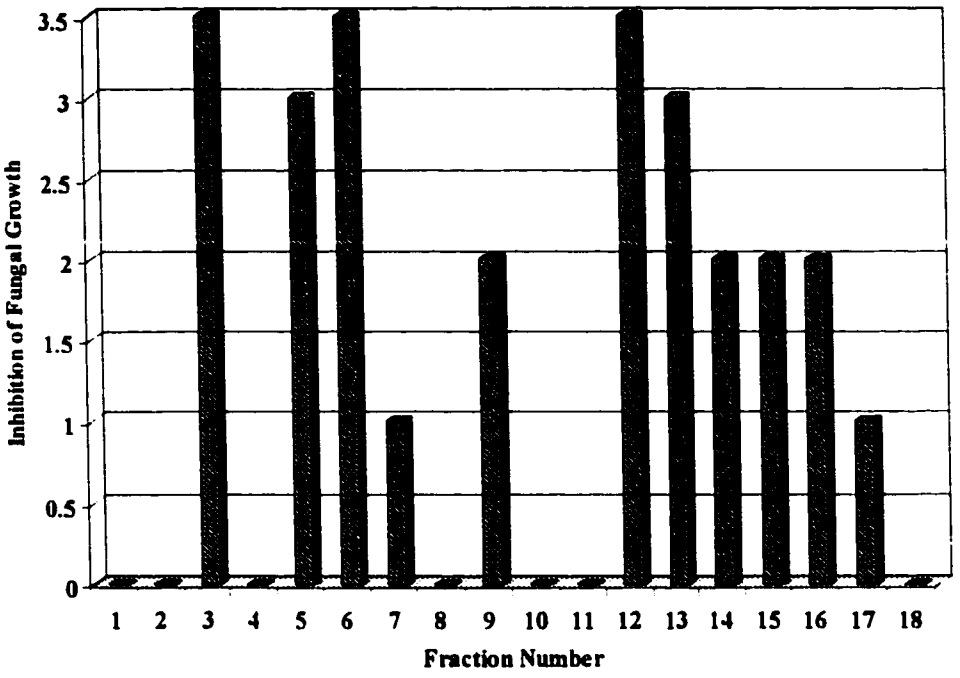


Figure V-16 : Antifungal Activity of Fractions Obtained after Anion Exchange Chromatography of Neutral Extract Superose 12 Fractions 4 and 5. Fractions from the RSQ column were dialyzed against 10 mM Tris-HCl, pH 8.0, and concentrated (2x) by ultrafiltration. One hundred µl of the concentrated sample from each fraction was assayed for antifungal activity using *A. flavus*.

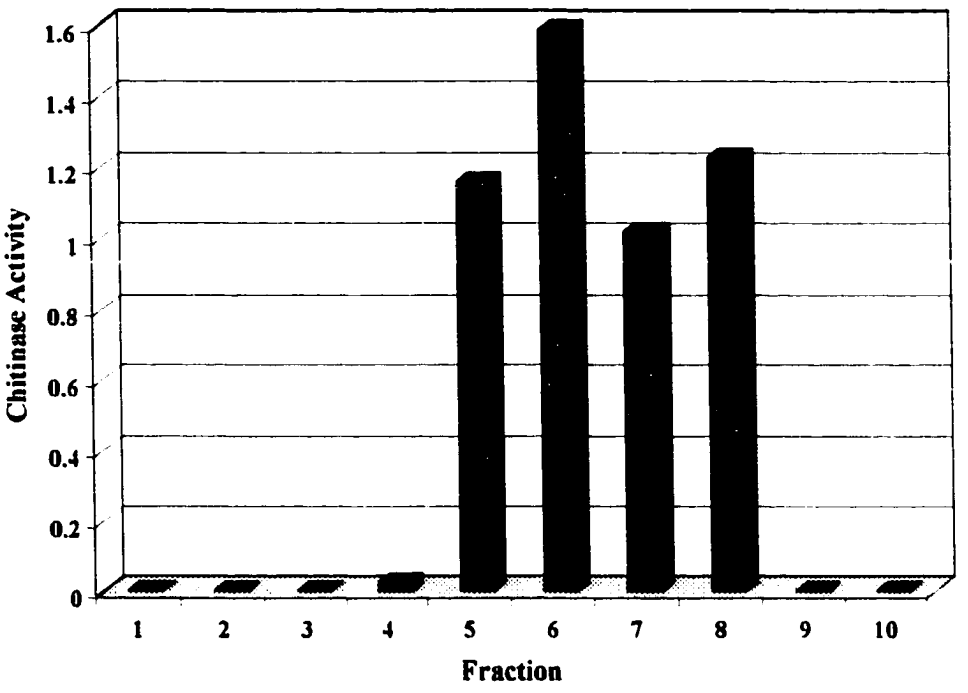


Figure V-17: Chitinase Activity of Fractions Obtained by Anion Exchange Chromatography of Superose 12 fraction 4 and 5. Fractions from anion exchange separation were dialyzed against 10 mM Tris-HCl, pH 8.0 and concentrated 2-fold by ultrafiltration. One hundred µl of the concentrated sample from each fraction was assayed for chitinase using the standard chitinase colorimetric assay. Because of the limited volume of fractions and amount of protein, fractions were pooled before assay. Fraction 1-4, RSQ fractions 1-4; fraction 5, RSQ fractions 5, 6 and 7; fraction 6, RSQ fraction 8; fraction 7, RSQ fraction 9; fraction 8, RSQ fraction 10; fraction 9, RSQ fractions 11-13; fraction 10, RSQ fractions 14-18.

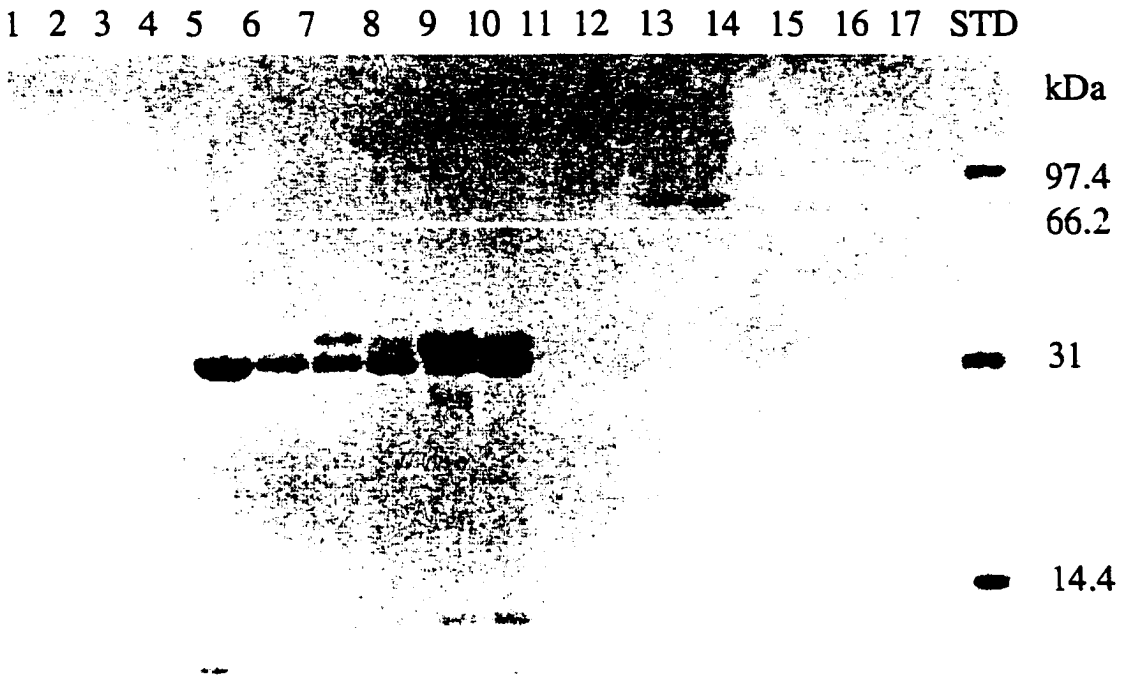


Figure V-18: SDS-PAGE Analysis of Fractions Obtained after Anion Exchange Chromatography of Superose 12 Fractions 4 and 5. Neutral extract was fractionated on a Superose 12 column. Superose 12 fractions 4 and 5 were pooled and the pooled fractions were applied to an anion exchange (RSQ) column. A 200 µl sample from each fraction was dried *in vacuo*, resuspended in 10 µl of SDS sample buffer and boiled for 5 min before loading onto the gel. Lane 1 to 17, RSQ fractions 1 to 17. STD = molecular weight standards.

and previous use of the Pharmacia RSQ column (a gift from Pharmacia, Upsala, Sweden) may account in part for the relatively poor resolution of individual components obtained in these separations.

Size-exclusion chromatography of fractions with hemagglutinating activity.

Fractions 9 and 10 from the neutral extract Superose 12 column contained several bands ranging in size from 20 to 41 kDa. Although not readily detected on the silver-stained SDS gels, there was evidence that lower molecular weight components (less than 14 kDa) may also be present in these active fractions. In an attempt to separate these components, a sample from fraction 9 was concentrated and subjected to size-exclusion chromatography on Sephadex G-50 resin (Figure V-19). Moderate to slight antifungal activity (Figure V-20) was observed in seven fractions (fractions 4-10). SDS-PAGE analysis of the G-50 column fractions (Figure V-21) revealed only one major protein band with a molecular weight near 21-24 kDa in fractions 8, 9 and 10. Protein bands were not detected in any other fraction.

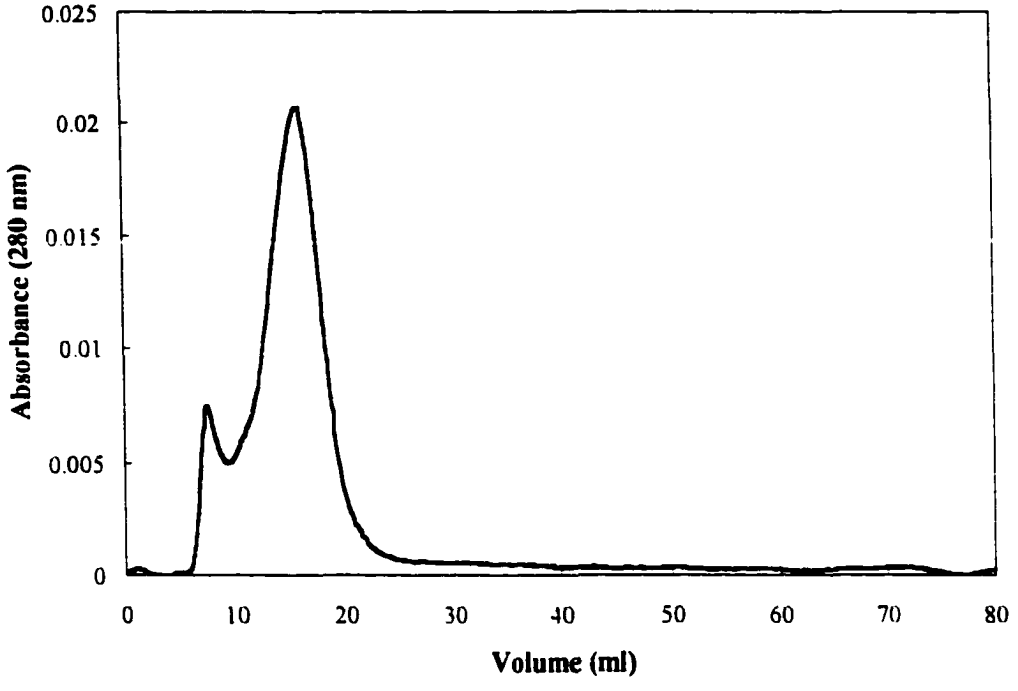


Figure V-19 : Sephadex G-50 Elution Profile of Neutral Extract Superose 12 Fraction 9. Pooled fraction 9 from several Superose12 runs was concentrated (4x) and 500 µl of concentrated fraction was separated on a G-50 column. Proteins were eluted with 10 mM Tris-HCl, pH 8.0. Fraction volume was 2 ml.

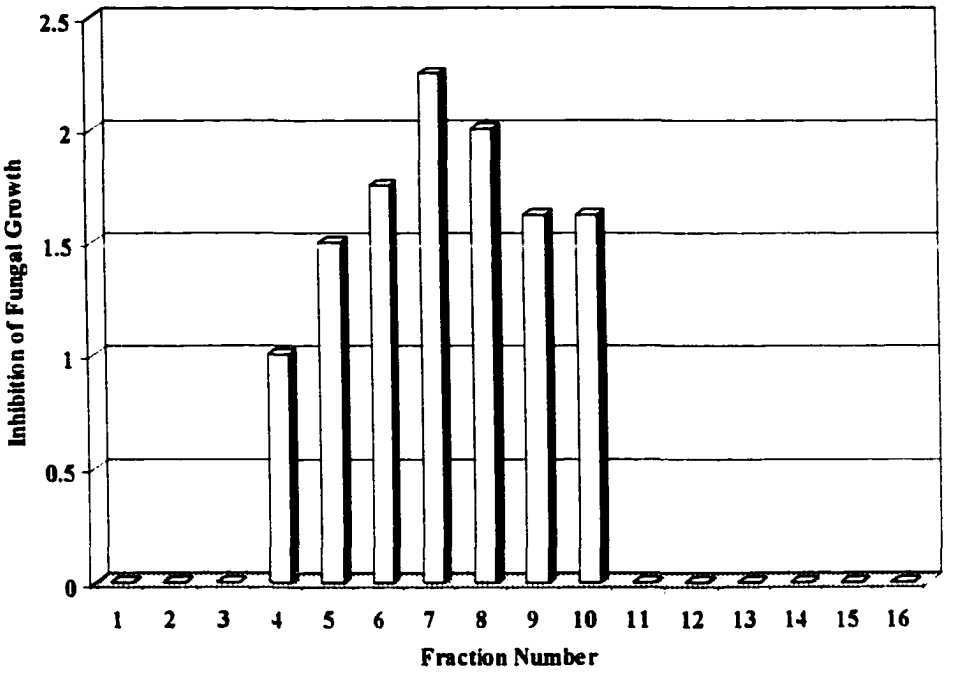


Figure V-20: Antifungal Activity of Fractions Obtained by Gel Filtration Chromatography of Neutral Extract Superose 12 Fraction 9 on Sephadex G-50. Superose 12 fraction 9 was concentrated and applied to a Sephadex G-50 column equilibrated with 10 mM Tris-HCl, pH 8.0. Fractions eluted from the column were tested for activity against *A. flavus*.

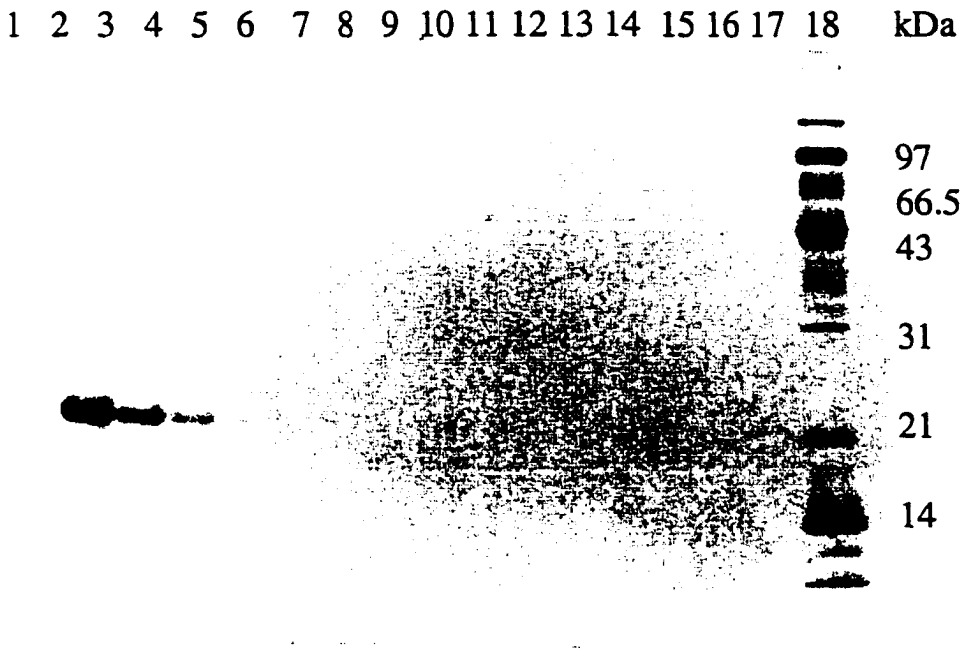


Figure V-21 : SDS-PAGE Analysis of Fractions Obtained by Gel Filtration Chromatography of Neutral Extract Superose 12 Fraction 9 on Sephadex G-50. Neutral extract was fractionated on a Superose 12 column. Fraction 9 containing antifungal activity and agglutinin was fractionated on a G-50 size-exclusion column. Lane 1 to 17, G-50 fractions 1 to 17. Lane 17, low molecular weight marker proteins. The gel was silver stained to reveal protein bands.

Ion-exchange chromatography of antifungal and chitinase-containing fractions from the acid extract Superose 12 column

Fractions 3-5 isolated by size-exclusion chromatography of the dialyzed acid extract exhibited antifungal activity as well as chitinase activity. These fractions were pooled and subjected to anion-exchange chromatography on a RSQ column for further purification of the antifungal and the chitinase activities. Fractions were collected and dialyzed against 10 mM Tris-HCl, pH 8.0. After dialysis, all of the fractions were assayed for antifungal activity (Figure V-22). Fractions 1, 4-7, 16 and 17 exhibited moderate to very strong antifungal activity. Analysis of the RSQ fractions by SDS-PAGE (Figure V-23) revealed two heavily stained bands of about 31-33 kDa in fractions 1 and 6-9. Although less intensely stained, bands of similar size were visible in fractions 2-5 and 10-14. Fractions 1 and fractions 6-7 possessed very distinct bands of molecular weight around 10-14 kDa. These bands were observed in the active fraction 4 and 5 but the intensity of

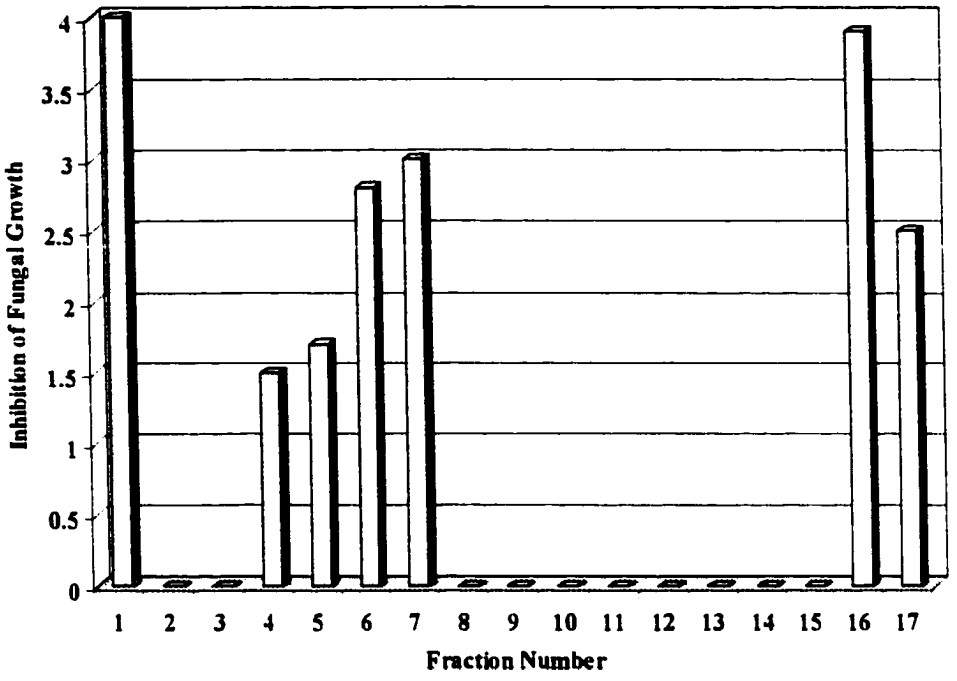


Figure V-22: Antifungal Activity of Fractions Obtained after Anion Exchange Chromatography of Chitinase-containing Fractions from the Acid Extract Superose 12 Column. Acid extract was fractionated on a Superose 12 column. Fractions 3 to 5 from the Superose 12 column were pooled and the pooled fractions were further fractionated by anion-exchange chromatography. Fractions from the anion exchange separation were dialyzed in 10 mM Tris-HCl, pH 8.0, and assayed for antifungal activity against *A. flavus*.

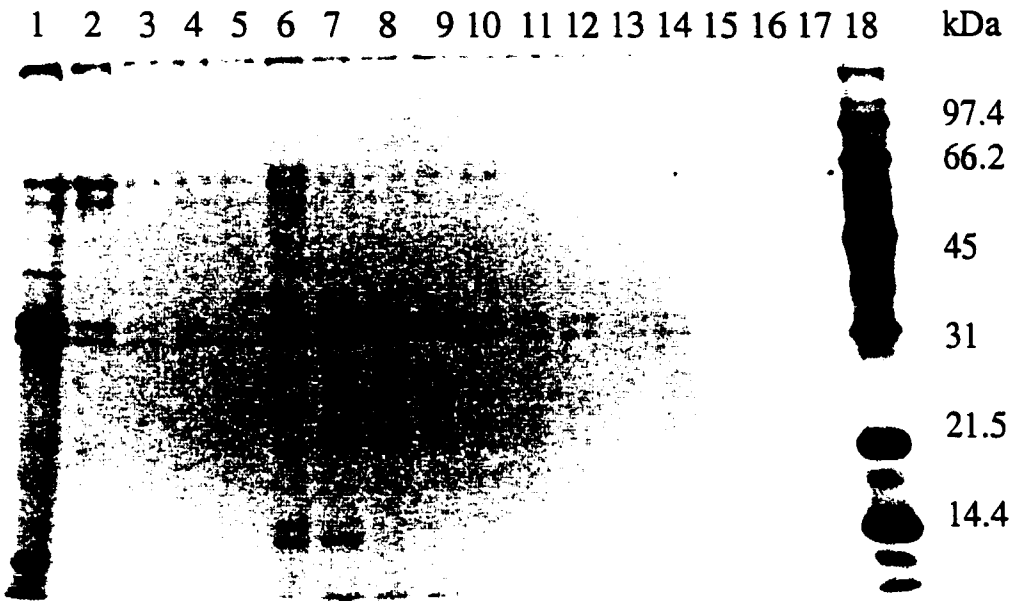


Figure V-23: SDS-PAGE Analysis of Fractions Obtained by Anion Exchange Chromatography of the Chitinase-containing Fractions from the Acid Extract Superose 12 Column. Acid extract was fractionated on a Superose 12 column. Superose 12 fractions 3 to 5 were pooled and the pooled fraction was applied to an anion exchange (RSQ) column. A 100 µl sample from each fraction was dried *in vacuo*, resuspended in 10 µl of SDS sample buffer and boiled for 5 min before loading onto the gel. Lane 1 to 17, RSQ fractions 1 to 17; lane 18, molecular weight marker proteins . The gel was silver stained to detect protein bands.

staining was substantially reduced. Several minor bands were also present in fraction 1 and fractions 6 through 9. Although fraction 16 and 17 exhibited antifungal activity, protein bands were not detected in these fractions.

PURIFICATION OF CHITINASE FROM EXTRACTS OF *S. SIMPLEX* SEEDS

A number of chitinases have been isolated from extracts of *S. simplex* seeds using a combination of chitin-affinity chromatography and anion-exchange chromatography. The results of the chitinase purification from the acetic acid eluate after separation of neutral extract by chitin-affinity chromatography are summarized in the form of a purification table (Table V-2). Results from two different separations are presented. Each of the individual chitinase-containing fractions may represent one or more isoforms of chitinase. This conclusion was confirmed after analysis of individual fractions for chitinase isoforms using a chitinase in-gel assay.

In the first fractionation, chitinases were separated into three pooled fractions (3, 4 and 5) using a steeper gradient with less resolution. In the

Table V-2: Summary of the Purification of Chitinase from Neutral Extracts of *S. simplex* Seeds.

Fraction	Activity	Protein	Volume	Specific Activity	Total Activity	Yield
	(μmol/h/ml)	(mg/ml)	(ml)	(μmol/h/mg)	(μmol/h)	(%)
Crude	7.7	1.56	5	5.0	38.7	100
ACP	5.0	0.42	1	12.0	20.0	51.7
First Purification						
3	0.094	0.012	1.96	7.8	1.8♦	4.7
4	0.126	0.02	1.97	6.3	2.5♦	6.5
5	0.057	0.072	1.97	0.79	1.1♦	2.8
Second Purification						
5	0.072	0.012	1.6	6.0	0.3♦	0.8
6	0.382	0.020	1.8	19.1	1.4♦	3.6
7	0.939	0.072	1.0	13.0	1.9♦	4.9
8	0.390	0.050	0.6	7.8	0.5♦	1.3
9	0.358	0.135	1.8	2.7	1.3♦	3.4
10	0.796	0.035	2.2	22.7	3.5♦	9.0

♦ Values normalized to account for volume applied to RSQ column.

second separation, the gradient was modified to increase resolution. Multiple forms of chitinase were detected in five fractions (5-10).

Note that the specific activity of the acetic acid eluate may be higher than some of the more purified fractions from the RSQ column. This apparent anomaly may be explained by the following. The acetic acid eluate although highly enriched for chitinase, is actually a mixture of chitinases and other proteins. The specific activity of this fraction represents the proportional sum of the activities of individual chitinases divided by the sum of the protein for each form of chitinase plus the concentration of other protein contaminants. Thus, the specific activity of individual fractions reflects a combination of the specific activities of individual isoforms of chitinase along with the proportion of the total chitinase and total protein represented by these isoforms. The presence of isoforms of chitinase with very high specific activity in the acetic acid eluate with subsequent loss of these forms, either totally or in part, after anion-exchange chromatography could account for the apparent anomaly. Chitinases represent a major part of the protein found in seeds *of S. simplex*. As a consequence, the fold purification was very low. The abundance of these proteins was evident in the crude extracts of *S. simplex* seeds.

CONCLUSIONS

Extracts of the seeds of *S. simplex* contain a variety of antifungal activities. Several of these are proteins while others may be peptides or other toxic molecules. The antifungal proteins may include chitinases, glucanases, lectins and chitin-binding proteins. Using a combination of chitin-affinity chromatography and anion-exchange chromatography, a number of chitinases have been purified. Some of these chitinases exhibit antifungal activity against *A. flavus* while others do not. Other antifungal activities were also isolated using this and other purification protocols. The biochemical nature of these activities is not yet known and will require further investigation.

Chapter VI : Characterization of Antifungal Proteins from Seeds of *Swartzia simplex*

INTRODUCTION

Several chitinases with antifungal activity were purified from extracts of seeds of *Swartzia simplex*. This work was discussed in Chapter V. These proteins account for only part of the antifungal activity contained in these extracts. Several isoforms of chitinase were further characterized. The results of this characterization are presented in this chapter.

MATERIALS AND METHODS

Glycoconjugate detection

To determine if the purified protein was glycosylated, samples from the ion-exchange column were tested using the Boehringer Mannheim DIG glycan detection kit. Proteins were first separated on a 12.5% SDS polyacrylamide gel under denaturing conditions. Proteins were transferred from the SDS gel to a nitrocellulose membrane according to standard methods used for Western blots. Proteins bound to the membranes were

oxidized with sodium metaperiodate and the oxidized proteins were reacted with DIG by incubating the nitrocellulose membrane as specified by the manufacturer. DIG-labeled proteins were detected with digioxigenin-specific antibody conjugated with alkaline phosphatase. The later was stained with NBT according to the manufacturer's protocol.

Amino acid analysis

A protein sample (after anion-exchange chromatography) was sent to the Molecular Biology Resource Center at the University of Oklahoma Health Sciences Center for amino acid analysis. The sample was purified by micro-HPLC on a reverse phase microbore column and the peak fraction was dried and hydrolyzed *in vacuo* in 6 N HCl for 24 h at 110 °C. The identity of the peak fraction after micro-HPLC was confirmed by SDS-PAGE. After hydrolysis, the amino acid composition was analyzed by cation-exchange chromatography using an automated Beckman System Gold Model 126 HPLC Amino Acid Analyzer. The amino acids were detected by on-line, post-column reaction with ninhydrin (Trione, Pickering Laboratories, Inc.).

Amino acid sequence analysis

Analysis of the N-terminal and internal sequence of purified proteins was performed at the Molecular Biology Resource Center at the University of Oklahoma Health Sciences Center. Purified protein samples were separated by micro-HPLC on a reverse phase column. The identity of the peak fraction was assessed by SDS-PAGE analysis of the micro-HPLC fractions. The fraction containing the protein band of interest was sequenced directly. The N-terminal and internal amino acid sequence was determined by Edman degradation using a PROCISE Model 492 protein sequencer equipped with an on-line PTH-amino acid analyzer (Perkin Elmer, Applied Biosystems Division) and model 610 data system.

The internal amino acid sequence of the purified protein was determined after digestion with LYS-C. The protease-digested samples were fractionated by micro-HPLC on a 1.0-mm x 150-mm C18 column to separate the peptides after protease digestion. The amino acid sequence of the internal peptides was obtained as described above.

Determination of molecular weight

The molecular weight of purified chitinase was determined by electrospray LC mass spectrometry. Mass spectrometry was performed by Dr. Ken Jackson at the Molecular Biology Resource Center using an API III triple quadrupole mass spectrometer (Sciex, Inc. Toronto, Canada) equipped with an atmospheric pressure ionization source.

Determination of isoelectric point

The isoelectric points of proteins in the chitinase-containing fractions from the RSQ column were estimated by conventional IEF gel electrophoresis. The purity and pI values for several of the purified proteins were also determined by capillary electrophoresis on a Beckman PACE 5000 capillary electrophoresis (CE) system using a CE-IEF kit, kindly provided by Dr. Bill Williams and Mr. Duncan Hare of Beckman Instruments, Inc., Palo Alto, CA.

Other methods are presented in detail in Chapter IV.

BIOCHEMICAL CHARACTERIZATION

Presence of chitinase isoforms

The presence of different isoforms of chitinase was determined using a chitinase in-gel assay. At least 8 different forms of chitinase were observed in the dialyzed neutral extract of *S. simplex* seeds (Figure VI-1). Five distinct isoforms were present in the neutral chitin column flow through and five forms were observed in the first Tris wash. Chitinase activity was not detected in the salt washes, 2nd Tris wash, acetic acid supernatant and sodium hydroxide supernatant. At least eight distinct isoforms were detected in the acetic acid eluate and five distinct isoforms were present in the sodium hydroxide eluate. The acetic acid eluate and the sodium hydroxide eluate were subjected to further purification by anion-exchange chromatography on a RSQ column.

SDS gel analysis of the RSQ fractions obtained after anion-exchange fractionation of the acetic acid eluate revealed one major protein band in fraction 5; fractions 6, 8 and 9 showed two bands each; fraction 7 had one major and three minor bands; and fraction 10 had three lightly-stained bands. With the exception of fraction 10, the most intensely stained bands in all these fractions, corresponded with the 31-kDa-protein marker. In

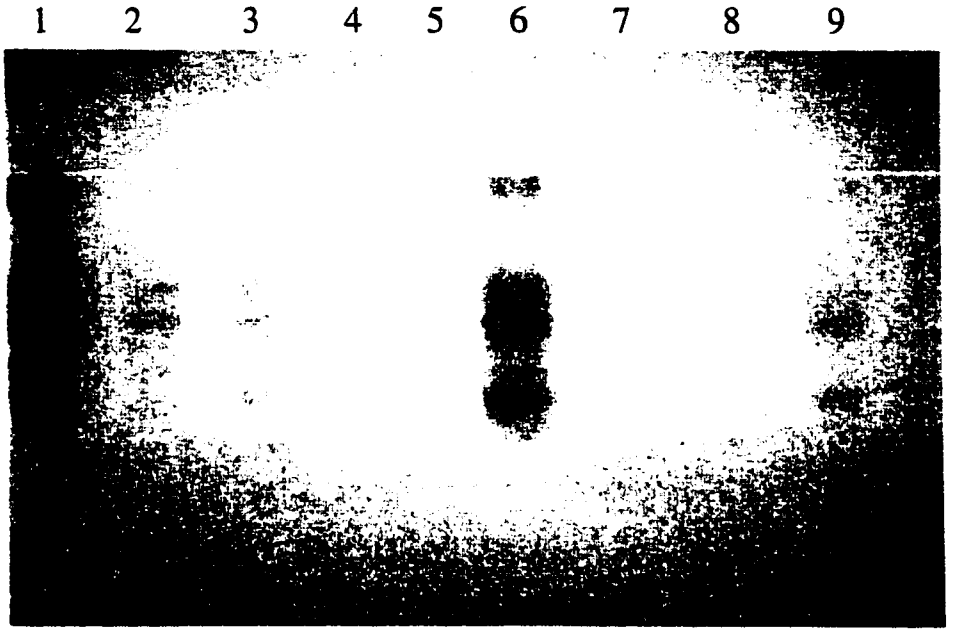


Figure VI-1 : Detection of Isoforms of Chitinases in Neutral Chitin Column Fractions. Neutral extract was fractionated on a chitin column. Ten µl from each fraction was separated on a 12.5% native gel. Chitinase activity was detected using an in-gel assay. Lane 1, neutral extract; lane 2, flow through; lane 3, 1st Tris wash; lane 4, salt wash (0.1 M); lane 5, salt wash (1 M); lane 6, acetic acid eluate; lane 7, acetic acid supernatant; lane 8, sodium hydroxide supernatant; lane 9, sodium hydroxide eluate.

In fraction 10, the second protein band was smaller than 31 kDa.

Results of the chitinase in-gel assay of the RSQ column fractions mentioned above are shown in Figure VI-2. Fractions 5, 6 and 7 each showed one form of chitinase. These were designated as *S. simplex* Chitinase A1 (fraction 5), A2 (fraction 6), and A3 (fraction 7). Fractions 8, 9 and 10 each had more than one form of chitinase. These chitinases differ in charge and/or size from each other and from A1, A2 and A3. Four chitinase bands were visible in fraction 8 (A4, A5, A6, and A7 in order of their increasing mobility), two chitinase bands (A8 and A9) were visible in fraction 9 and two chitinases (A10 and A11) were detected in fraction 10. Chitinase bands in fractions 5, 6, and 7 were broad and diffuse. For this reason, it was difficult to determine if there was more than one form of chitinase present in these fractions.

At least five different forms of chitinase were present in the sodium hydroxide eluate. The latter was further fractionated on an RSQ column. Three of these purified isoforms were detected in an activity gel of the fractions obtained by anion-exchange chromatography of sodium hydroxide eluate (Figure VI-3). Two chitinase bands were observed in fraction 4, and a single band was detected in fraction 5. These bands were designated as *S. simplex* chitinase B1 and B2 (fraction 4), and B3 (fraction 5).

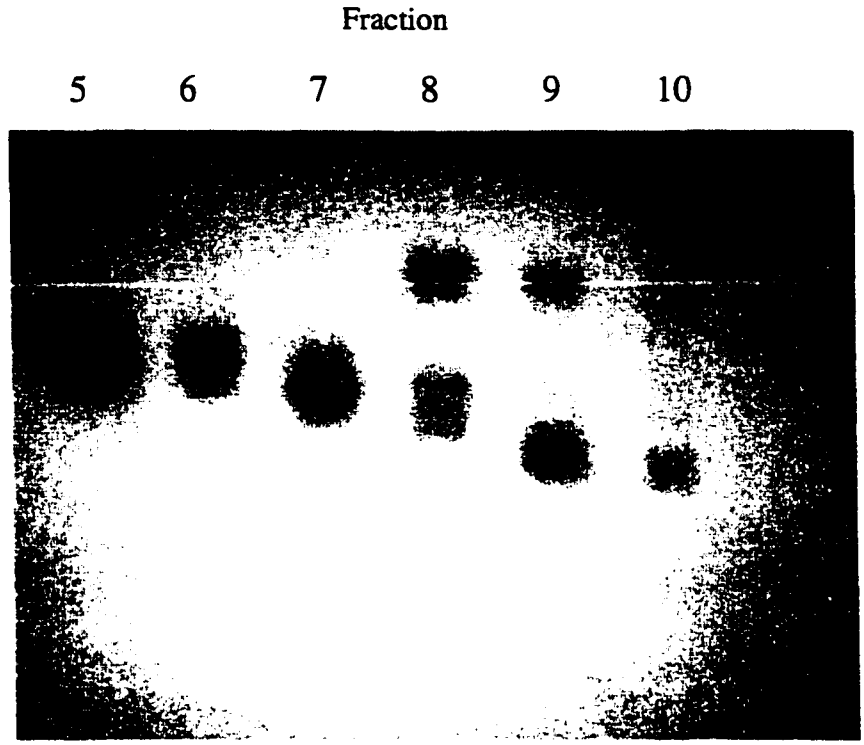


Figure VI-2: Detection of Isoforms of Chitinases in the RSQ Column Fractions 5-10 (see Figure V-3) from the Neutral Chitin Column Acetic Acid Eluate. Acetic acid eluate from the neutral chitin column was separated by anion exchange (RSQ) chromatography. Fractions were dialyzed against 10 mM Tris-HCl, pH 8.0, to remove salt. A sample from each of the fractions was concentrated and 10 µl of the concentrated sample was separated on a 12.5% native gel. Chitinase activity was detected using an in-gel assay.

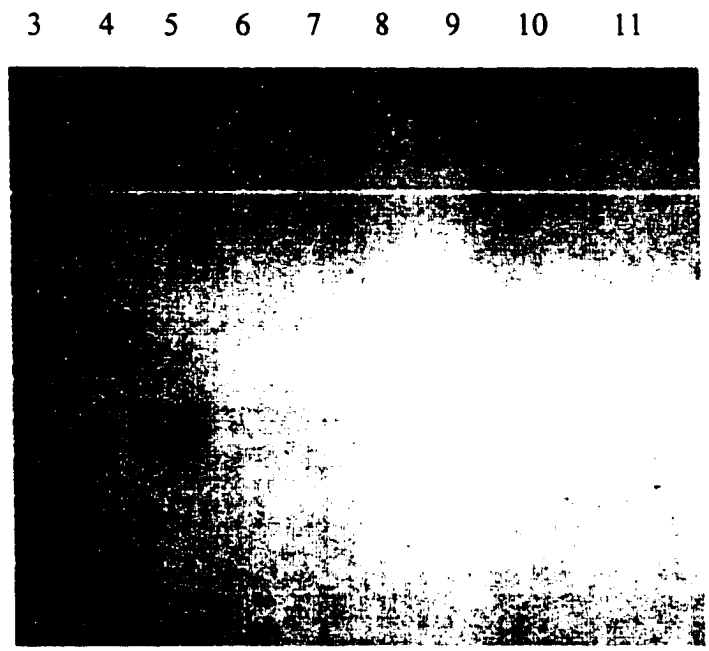


Figure VI-3: Isoforms of Chitinase in Fractions Obtained by Anion Exchange Chromatography of the Sodium Hydroxide Eluate from a Neutral Chitin Column. A sample from each fraction was concentrated and 10 µl of the concentrated sample was separated on a 12.5% native gel. Chitinase activity was detected using the in-gel assay. Fractions refer to RSQ fractions 3-11 as indicated in Figure V-7.

Detection of β–Glucanase by Glucanase In-Gel Activity Assay: The presence of β-glucanase in the *S. simplex* dialyzed neutral extract and dialyzed neutral extract chitin column fractions was determined using the glucanase in-gel activity assay. All the fractions used for the in-gel assay were concentrated (2x) and 15 μl of each of the concentrated sample was separated on a 12.5% polyacrylamide gel under non-denaturing conditions. After electrophoresis, the gels were incubated in laminarin for 30 min. Glucanase bands were detected after color development as described in the Materials and Methods (Chapter IV).

Dialyzed neutral extracts contained different forms of glucanases. At least four different bands were visible in the neutral extract of *S. simplex* seeds. However, two bands were detected in the acetic acid eluate and no bands were visible in the flow through or the sodium hydroxide eluate (Figure VI-4). Apparently, some forms of glucanase were lost after chitin column chromatography. The possibility exists that other forms of glucanase were present in these chitin column fractions but at levels below the levels of detection using the in-gel assay. In the future, alternative methods of purification of glucanases may be more productive.

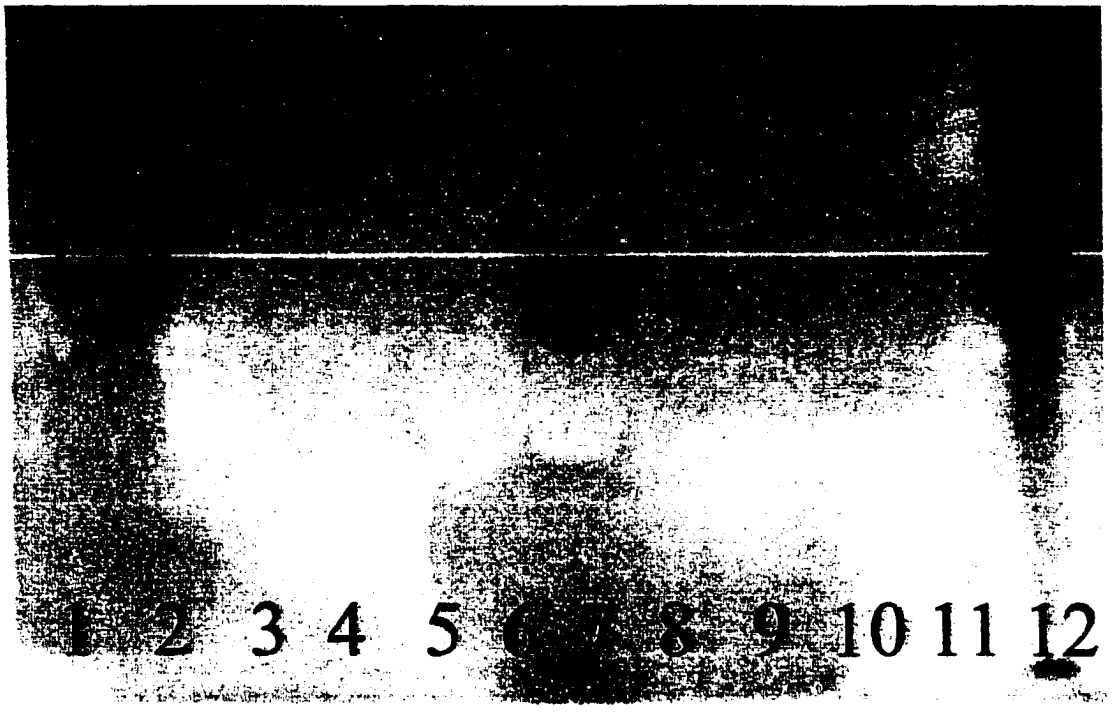


Figure VI-4: Glucanase In-Gel Assay of Neutral Chitin Column and RSQ Fractions. Samples were concentrated (2x) and 15 µl of each of the concentrated samples was separated on a 12.5% native gel. After electrophoresis, glucanases were detected using the glucanase in-gel assay. Lane 1, *S. simplex* neutral extract; lane 2, flow through from neutral chitin column; lane 3, neutral chitin column sodium hydroxide eluate; lane 4; neutral chitin column acetic acid eluate; Lane 5-11; RSQ fractions 5-11 obtained by anion-exchange chromatography of the neutral chitin column acetic acid eluate. Lane 12; positive control.

Detection of Protein Glycosylation

The glycosylation of purified chitinases obtained by anion-exchange chromatography of the neutral chitin column acetic acid eluate was measured using a DIG glycan detection system. Two hundred µl of each fraction was dried in a speed vac and 10 µl of SDS sample buffer was added to each of the dried samples. After heating in a boiling waterbath for five min, these samples were separated on a 12.5% SDS polyacrylamide gel and then transferred onto a nitrocellulose membrane. All the chitinase bands were stained indicating the presence of carbohydrates attached to these proteins (Figure VI-5). In addition, glycosylation was also observed in fraction 14 from the RSQ column even though there was no visible protein band in this fraction.

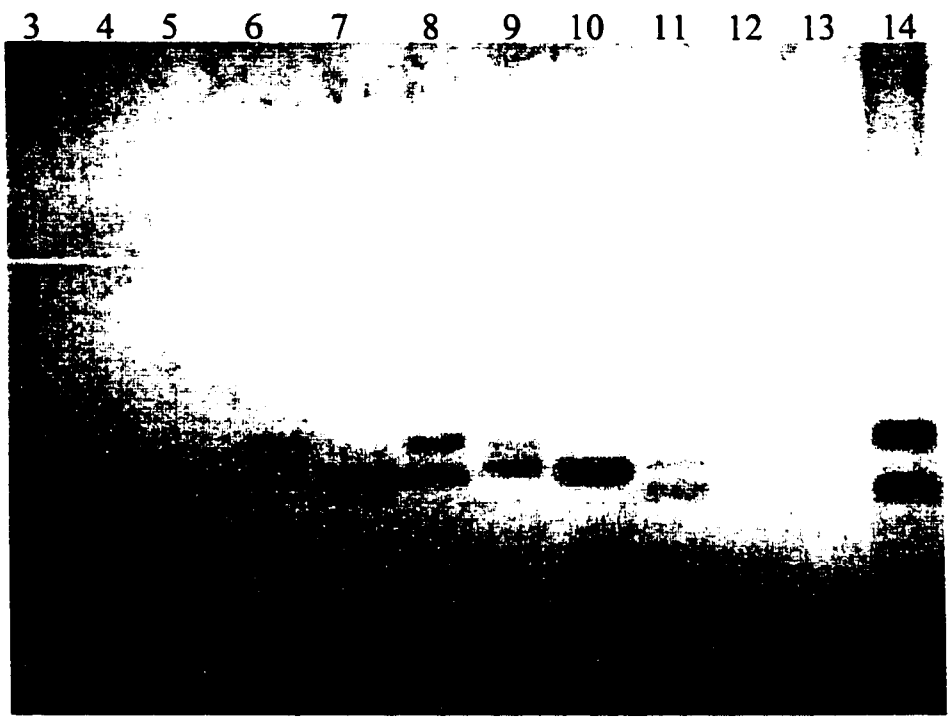


Figure VI-5: Glycoprotein Analysis of Ion Exchange Column (RSQ) Fractions of Acetic Acid Eluate from the Neutral Chitin Column. Glycoprotein analysis was performed after SDS-PAGE separation. Proteins were transferred to a nitrocellulose membrane. Glycoproteins were detected using the Boehringer Mannheim DIG glycan detection kit. Lane 3 to 14, RSQ column fractions 3 to 14, respectively.

Amino Acid Composition

Purified *S. simplex* chitinase A 3 (fraction 7 from the RSQ column) was loaded onto a micro-HPLC reverse phase column to remove minor contaminants before analysis of amino acid composition or sequence. The absorbance profile indicated two peaks (Figure VI-6), a major peak in fraction 8 with a trailing edge in fraction 9 and one minor peak in fraction 4. SDS-PAGE analysis confirmed the identity of the peak 8 (Figure VI-7). Fraction 8 was dried under vacuum and part of this sample was subjected to acid hydrolysis to determine its amino acid composition using a auto analyzer (Table VI-1).

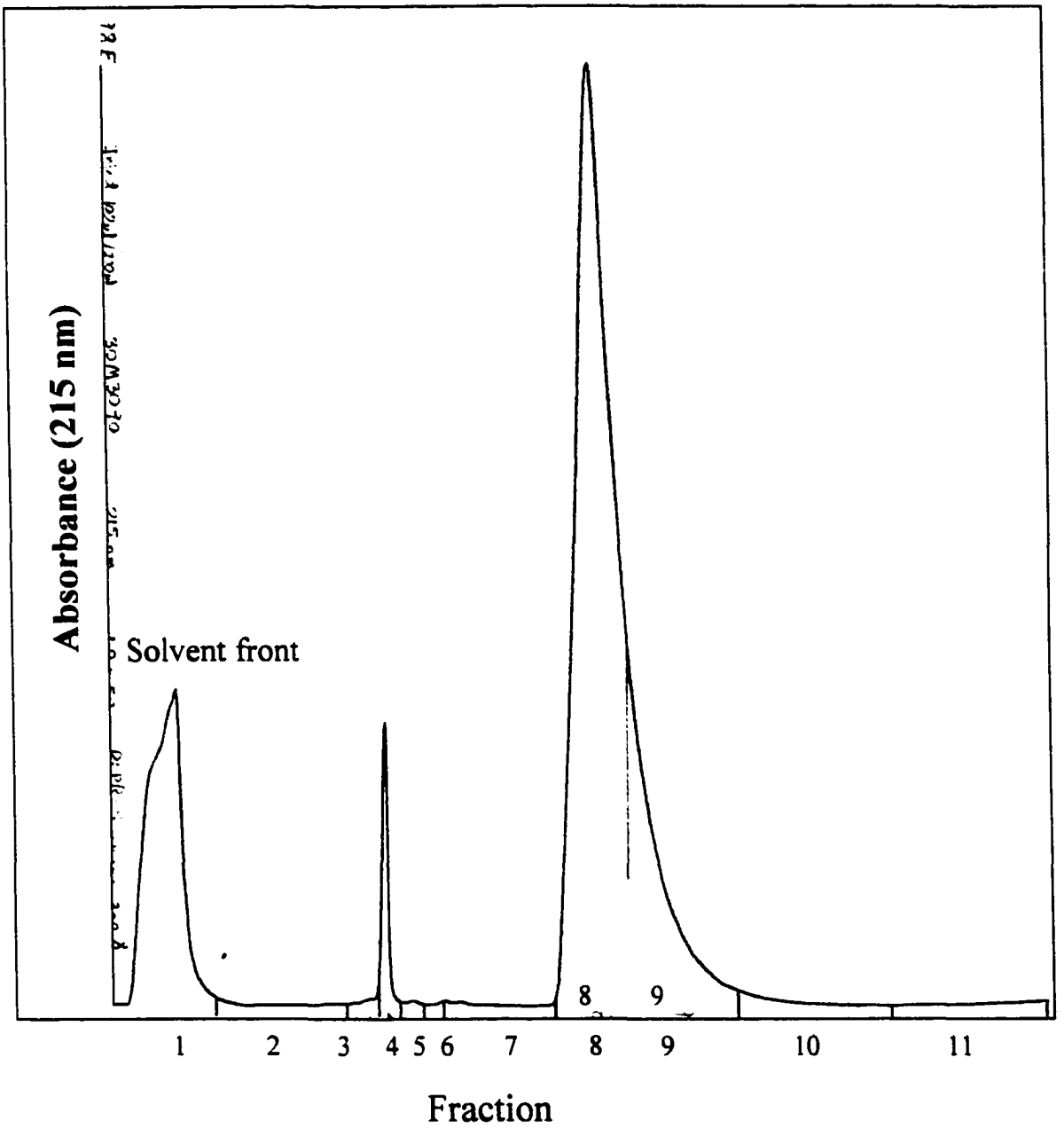


Figure VI-6: Absorbance Profile of HPLC Separation of Chitinase A3 Prior to Sequencing. Prior to HPLC purification, 72 µl of RSQ fraction 7 containing chitinase A3 was added to 128 µl of TFA in water. One hundred µl of sample was injected onto a 1.0 mm x 50 mm 200 A° PLPR-S column. the column was eluted with a gradient of acetonitrile-water containg TFA. Peak fractions were collected manually.

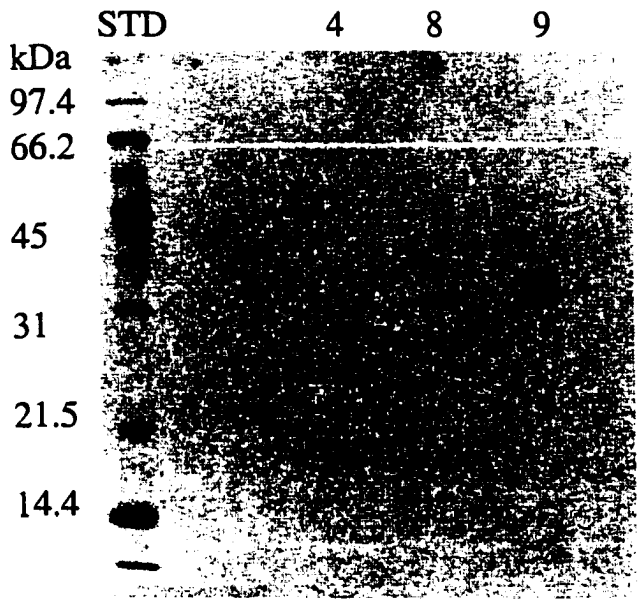


Figure VI-7: SDS-PAGE Analysis of Fractions from Micro-HPLC Separation of Chitinase A3. Acetic acid eluate from the neutral chitin column was fractionated by anion exchange (RSQ) chromatography. Fraction 7 from the RSQ column containing chitinase A3 was further purified by micro-HPLC prior to sequencing. Fraction 4, 8 and 9 (see Figure VI-6) were dried and analyzed by SDS-PAGE. The protein banding pattern of the fractions corresponding to the peaks are shown on the figure. After electrophoresis, the gel was silver stained to reveal protein bands. STD = molecular weight standards.

Table VI-1: Amino Acid Composition of Chitinase A3.

AMINO ACID	NUMBER OF RESIDUES
Aspartic acid	46.0
Threonine	11.0
Serine	22.5
Glutamic acid	18.6
Proline	15.7
Glycine	29.2
Alanine	20.5
Cystine	2.9
Valine	14.1
Methionine	1.2
Isoleucine	13.1
Leucine	26.6
Tyrosine	15.3
Phenylalanine	12.3
Lysine	15.1
Histidine	7.1
Arginine	6.0
TOTAL	273.1

Amino acid analysis of chitinase A3 [Fraction 7]was performed on an automated amino acid analyzer after acid hydrolysis of the HPLC purified protein.

Amino Acid Sequence Analysis

The N-terminal sequences of several of the purified chitinase isoforms were analyzed using a ProCise Automated Sequencer. The N-terminal sequence of both HPLC fractions 8 and 9 described above was determined. The two sequences obtained were identical, suggesting that the protein present in fractions 8 and 9 was the same even though two bands were detected on the SDS gel of fraction 9. Glycosylation may have been responsible for the trailing edge of the peak and the appearance of multiple bands in this fraction. Alternatively, the N-terminus of the higher molecular weight form may have been blocked. The N-terminal sequence of *S. simplex* chitinase A1 (fraction 5) was the same as the N-terminal sequence of chitinase A3 (Figure VI-8).

Although these proteins share the same N-terminal sequence, they must differ in internal sequence since they can be separated by ion-exchange chromatography and native gel electrophoresis. The N-terminal sequence of A10/11 (fraction 10) was similar but not identical to the sequences of chitinase A1 and A3 (Figure VI-8). The N-terminal amino acid sequences of

Figure VI-8: N-terminal Sequence of *S. simplex* Chitinases. The sequences were determined by Edman degradation with an automated protein sequencer. No amino acid residue was detected in position 23 of all three chitinases.

Chiti A1	KVNGWITIYWGQNNGDGTLAST?DTGLYEI
Chiti A3	KVNGWITIYWGQNNGDGTLAST?DTGLYEIVNLAF
Chiti A10/A11	RVDGWITIYWGQNNGDGSLTAT?DTGLYNI

chitinase A3 and A10/11 were compared to the N-terminal sequences of plant chitinases from different species using the Swiss-Pro Gene Bank database through the Internet (Figure VI-9 and Figure VI-10). The chitinase A3 N-terminal sequence was highly homologous to class III acidic endochitinase/lysozyme precursor from several plant species (Figure VI-9). Over 60% of the N-terminal sequence of chitinase A10/11 was identical to the N-terminal sequences of several class III chitinase/lysozyme and class III chitinase/lysozyme precursors from different plants (Figure VI-10 and Figure VI-11), including *Cucumis sativus* (cucumber).

To obtain the internal sequence for chitinase A3, the most prominent chitinase, the HPLC purified A3 (fraction 8) was digested with Lys-C (Boehringer-Mannheim, Indianapolis IN). The peptide fragments obtained after the digestion were purified by RP-HPLC (Figure VII-12). Eight major peaks were separated and the N-terminal sequence of three of these peaks was determined. The internal sequences obtained are presented in Figure VII-13. Comparison of the internal sequences of chitinase A3 to cucumber class III endochitinase precursor indicated over 50% sequence homology (Figure VI-14).

Figure VI-9: N-terminal Sequence Homology of *S. simplex* Chitinase A3 to Chitinases from Other Plant Species.

Plant Species	Access number *	% Identity	% Similarity	References
Cucumis sativus	p17541	64	73	Metraux et al., 1989
Phaseolus angularis	p29024	51	68	Ishige et al., 1993
Vitis vinifera	p51614	57	68	Busam et al., 1996
Parthenocissus quinquefolia	p23473	57	63	Bernasconi et al., 1987
Nicotiana tabacum	p29061	54	63	Lawton et al., 1992
Arabidopsis thaliana	p19172	54	66	Samac et al., 1990
Nicotiana tabacum	p29060	54	63	Lawton et al., 1992
Hevea brasiliensis	p23472	54	60	Jekel et al., 1991
Cicer arietinum	p36908	46	64	Vogelsang and Barz, 1993
Beta vulgaris	p36910	64	75	Nielsen et al., 1993

* Swiss-Pro protein access number

Figure VI-10: N-terminal Sequence Homology of *S. simplex* Chitinase A10/11 to Chitinases from Other Plant Species.

Plant species	**Access number** *	**% identity**	**% similarity**	**Reference**
Parthenocissus quinquefolia	p23473	63	73	(Bernasconi et al., 1987)
Vitis vinifera	p51614	63	73	(Busam et al., 1996)
Hevea brasiliensis	p23472	63	73	(Jekel et al., 1991)
Phaseolus angularis	p29024	62	81	(Ishige et al., 1993)
Arabidopsis thaliana	p19172	63	78	(Samac et al., 1990)
Beta vulgaris	p36910	64	70	(Nielsen et al., 1993)
Nicotiana tabacum	p29061	52	68	(Lawton et al., 1992)
Cucumis sativus	p17541	64	76	Metraux et al., 1989
Nicotiana tabacum	p29060	63	68	(Lawton et al., 1992)
Cicer arietnum	p36908	64	78	(Vogelsang and Barz, 1993)

* Swiss-pro protein access number.

Figure VII-11: Comparison of the N-terminal Sequence of *S. simplex* Chitinase A10 with N-terminal Sequences of Other Plant Chitinases.

```
CHIA 10          RVDGWITIYWGQNNGDGSLTAT?DTGLYNI
                   * * ******* + * +*+* *
CHIA ARATH         GGIAIYWGQNGNEGNLSAT
CHLY PARTH         GGIAIYWGQNGNEGTLTQT
CHIA VITVI         GGIAIYWGQNGNEGTLTQT
CHLY HEVBR         GG IAIYWGQNGNEGTLTQT
```

CHIA ARATH = acidic endochitinase precursor from *Arabidopsis thaliana;* CHLY PARTH = chitinase / lysozyme from *Parthenocissus quinquefolia.*; CHIA VITVI = acidic endochitinase precursor from *Vitis vinifera*; CHLY HEVBR = chitinase / lysozyme from *Hevea brasiliensis*. (*) = identical, (+) = similar.

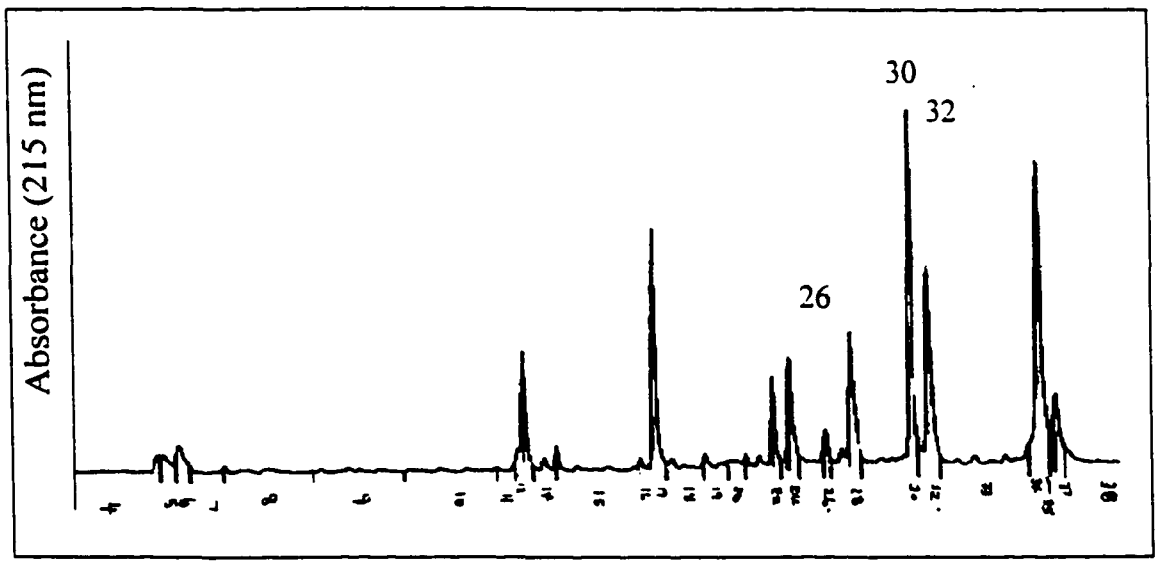


Figure VI-12 : HPLC Separation of Products of Lys-C Digestion of Chitinase A3. To obtain internal sequence of chitinase A3, chitinase A3 was digested with Lys-C and the products of digestion were separated on a 1.0-mm x 150-mm C18 column.

Figure VI-13: Internal Sequence of *S. simplex* Chitinase A3. The internal sequences were determined after micro-HPLC purification of the LYS-C digested fractions of Chitinase A3. No amino acids were detected in position 31 and 34 of LYS-C digested Chitinase A3 fraction 30.

Fraction 26 YYLSAAPQCFIPDYYLDK

Fraction 30 TGLFDDIFVQFTNNPPCQYASGDPDRLFQS?DA?T

Fraction 32 YGPLGSVALDGIDFDIQGGSNLYWDDLVRGLDTLRK

Figure VI-14: Comparison of Homology between *S. simplex* Chitinase A3 LYS-C Digested Fractions and Cucumber Class III Acidic Chitinase.

HPLC Fraction	% Identity	% Similarity
26	80	86
30	70	85
32	55	72

Reference: Metraux et al., 1989

Determination of molecular weight

The molecular weights of the chitinases from *S. simplex* were determined by SDS-PAGE (Figure VI-15). Samples from the chitinase containing fractions were reduced with β–mercaptoethanol and boiled for 5 min before loading onto the gel. Based on this analysis, the subunit molecular weight of the major protein bands ranged from 28 kDa to 36 kDa. There were a few minor bands present in most of the chitinase-containing fractions (Chit A1 to A11). The molecular weights of all these protein bands are summarized in Table VI-2.

The molecular weight of *S. simplex* chitinase A3 (RSQ fraction 7) was also determined by LC mass spectrometry at the University of Oklahoma HSC Molecular Biology Resource Center. Three proteins that were closely related in molecular weight were observed in the HPLC purified *S. simplex* chitinase A3. The molecular weight of the major peak was 32,309 Da and the two smaller peaks were 32,151 Da. and 32,478 Da (Figure VI-16). The heterogeneity in molecular mass was most likely due to differences in glycosylation. Consistent with this conclusion, the differences in weight between the three peaks were approximately 162 Da (the residue weight of an additional hexose unit).

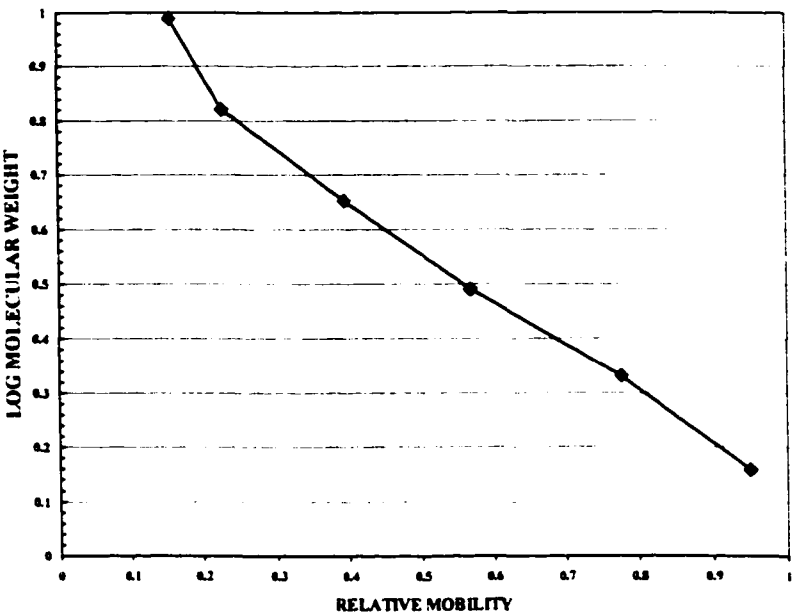


Figure VI-15: Estimation of Protein Molecular Weights by SDS-PAGE. Plot of log Molecular weight of protein standards versus relative mobility on a 12.5% SDS gel.

Table VI-2: Estimated Molecular Weights of Purified Chitinases and Other Minor Protein Bands Present in the Chitinase-containing Fractions Obtained by Anion-exchange Chromatography of the Acetic Acid Eluate from the Neutral Chitin Column.

Fraction number	Band	Molecular weight (kDa)
5	major	33
6	major 1	36
	major 2	34
7	major	37
	minor 1	33
	minor 2	30
	minor 3	28
8	major 1	36
	major 2	34
9	major	36
	minor	35
10	minor 1	36
	minor 2	34
	minor 3	28

Molecular weights of chitinase were determined by SDS PAGE. Numbers were assigned according to the mobility of proteins on the SDS gel. The highest numbers were assigned to the proteins with highest mobility.

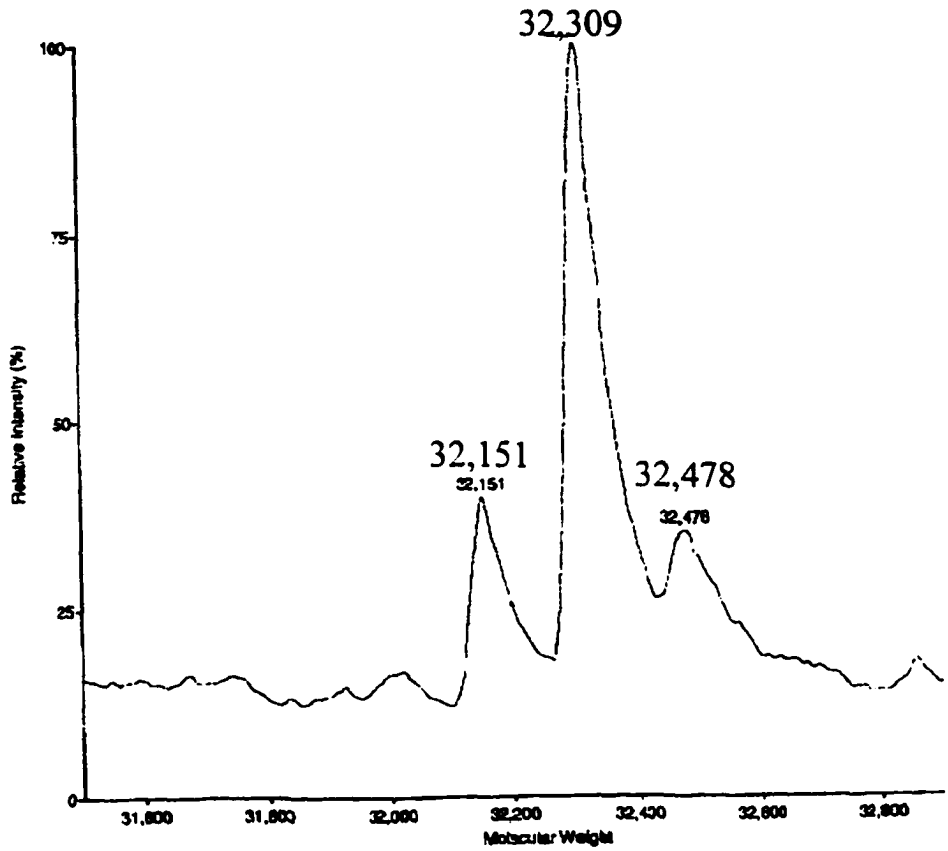


Figure VI-16: Determinationof Molecular Weight of Chitinase A3 by Electrospray Mass Spectrometry.

Isoelectric point

The isoelectric points of several of the purified *S simplex* chitinases were determined by isoelectric focusing using a BioRad mini-IEF chamber. The IEF gel was prepared according to the manufacturer's instructions. After the sample was focused, the gel was stained with Coomassie Brilliant Blue. The pH profile was estimated by measuring the migration distance from the anode for a series of pI standards from BioRad. Results of this analysis indicate that the major pI's of the chitinase containing bands were very acidic with pI's below 5.2 (data not shown).

Capillary IEF was performed with *S. simplex* chitinase A1, A3 and A10/11 using a Beckman PACE 5000 system. Based on the results of this analysis, the isoelectric point of *S. simplex* chitinase A3 was 3.0 (Figure VI-17).

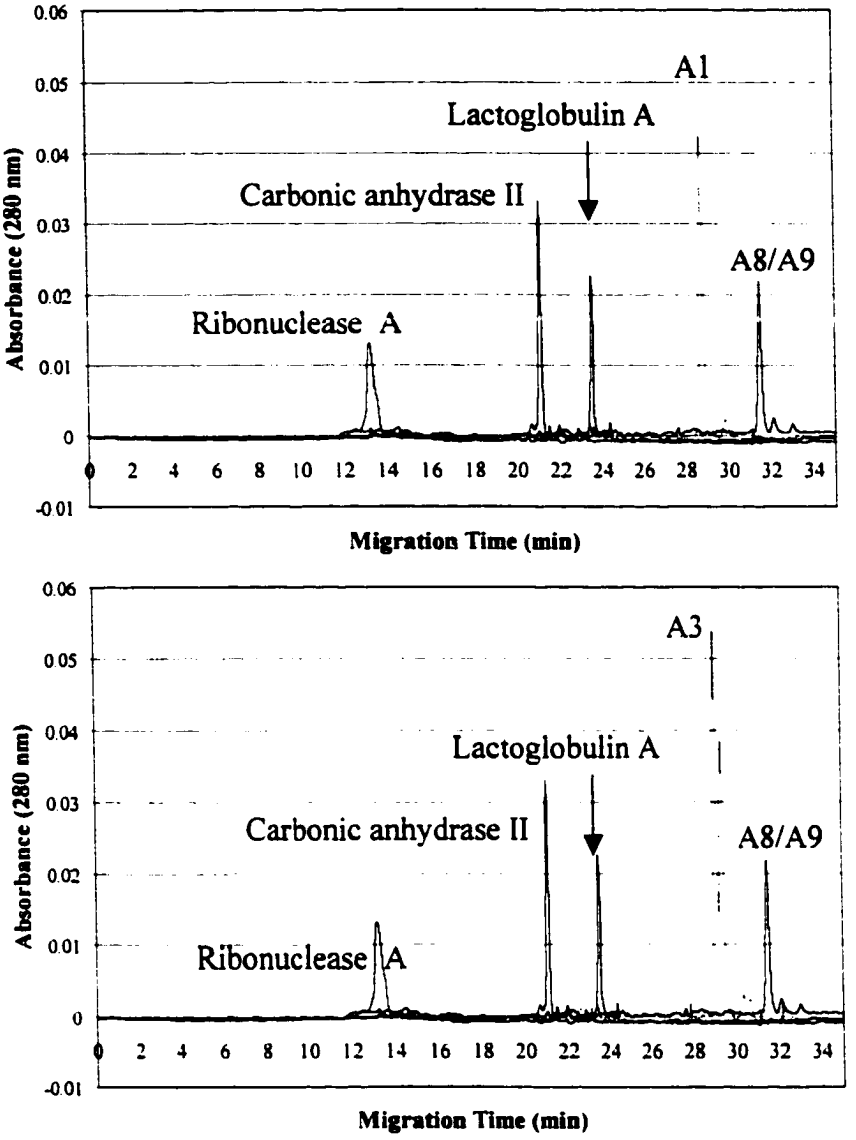


Figure VI-17: Determination of Isoelectric Points for Chitinases from *S. simplex* by Capillary Isoelectric Focusing (CEIEF). Samples with standards were separated by CEIEF on a Beckman PACE 5000. Standards include ribonuclease A, carbonic anhydrase II and β–Lactoglobulin A with pI values of 9.45, 5.90 and 5.10, respectively. Position of standards and samples are noted on the figures. RSQ fraction 5 = chitinase A1; fraction 7 = chitinase A3; fraction 9 = chitinase A8/A9.

CONCLUSIONS

A number of isoforms of chitinase were detected in extracts from *S. simplex* seeds. These chitinases were purified as described in Chapter V. The properties of several of these chitinases are reported in this chapter. The chitinases have molecular weights in the range of 30-32 kDa based on mass spectrometry and SDS-PAGE. The isoelectric points were determined for several isoforms using conventional and capillary IEF. These proteins were all acidic with pI values near 3. A partial amino acid sequence was determined for three of the chitinases isolated by anion-exchange chromatography. These chitinases have high sequence homology with class III chitinase/lysozyme from other plant sources. Based on results of an antifungal bioassay, several of these chitinases exhibit antifungal activity against *A. flavus*. Further analysis and gene cloning will be required to determine the structures of all of these chitinases and to determine their role in plant defense.

Chapter VII : Purification and Characterization of Antifungal Activities from *Swartzia cubensis*

INTRODUCTION

A closely-related species of *Swartzia* known as *Swartzia cubensis* (Britt. & Wils.) Standl. was collected by Dr. Karel Schubert from the lowland humid forests of Costa Rica. This species was not included in the initial screen. When tested for antifungal activity, both crude and dialyzed extracts of *S. cubensis* seeds completely inhibited conidial germination and growth of *F. moniliforme* and showed very strong inhibition of *A. flavus* hyphal growth. Since both species apparently contain potent inhibitors of fungal growth, the purification of antifungal activities from *S. cubensis* and *S. simplex* seeds was carried out in parallel. Although the same process for development of a purification method as presented in Chapter IV was used herein, only the results of selected experiments will be presented.

MATERIALS AND METHODS

Seeds of *Swartzia cubensis* were collected from the lowland rainforest of Costa Rica and stored at –40°C. The general methods used for the isolation of antifungal activities from extracts of *S. cubensis* seeds were the same, unless otherwise noted, to those used for the purification of antifungal activities from *S. simplex.*

Ammonium sulfate fractionation

Solid ammonium sulfate was pulverized with a mortar and pestle and gradually added to a sample of neutral crude dialyzed extract to achieve 25% saturation of ammonium sulfate. The procedure was carried out on ice with constant stirring. After all of the salt dissolved, the sample was kept on ice for 60 min without stirring and then centrifuged for 30 min at 15,000 x g. The pellet was resuspended in a minimum volume of 10 mM Tris-HCl (pH 8.0). Additional ammonium sulfate was added to the supernatant to a final concentration of 35% saturation and the process described above was repeated, for the 25-35% ammonium sulfate saturated sample. The supernatant obtained after this step was adjusted with the addition of more solid ammonium sulfate and the process was repeated to obtain fractions for

35-45, 45-55, 55-65 and 65-90% ammonium sulfate saturation. In each case, the pellet obtained after each fractionation was resuspended in 10 mM Tris-HCl (pH 8.0). The supernatant remaining after the final precipitation along with the resuspended pellets obtained after each step of the ammonium sulfate fractionation were dialyzed in 10 mM Tris-HCl (pH 8.0) and assayed for antifungal activity.

PRELIMINARY CHARACTERIZATION AND PROPERTIES OF ANTIFUNGAL ACTIVITY.

Heat stability

Samples of dialyzed neutral extract from *S. cubensis* seeds were heated at 50, 60, 70, 80, 90 and 100 °C for 3 min and the heat-treated extracts were assayed for antifungal activity. Results of the bioassay indicated that antifungal activity was denatured by heat treatment, i.e. there was no inhibition of *A. flavus* growth in heat-treated samples. The extreme sensitivity of the antifungal activity to heat supports the conclusion that the active components are proteins.

Size fractionation by ultrafiltration

To estimate the size of the active components in these extracts, dialyzed neutral extract was size fractionated by ultrafiltration using Centricon C10, C30, C50 or C100 concentrators. The retentates and filtrates after ultrafiltration were tested for antifungal activity. The results of these bioassays were very similar to the results obtained with *S. simplex* extracts. After 48 h of incubation, all of the retentates completely inhibited conidial germination of *A. flavus* (Figure VII-1). Moderate inhibition of hyphal growth was observed with all the filtrates in the 48-h assay but the activity diminished with increased time of incubation. After 7 days of incubation, heavy mycelial growth was observed in all the filtrates while all the retentates still showed complete inhibition. These results suggest that components with molecular weight greater than 10 kDa possess antifungal activity. In addition to the retentates, the filtrates apparently possess antifungal activity. Inhibition of fungal growth by these filtrates, however, was not complete and fungal growth was evident only after prolonged incubation.

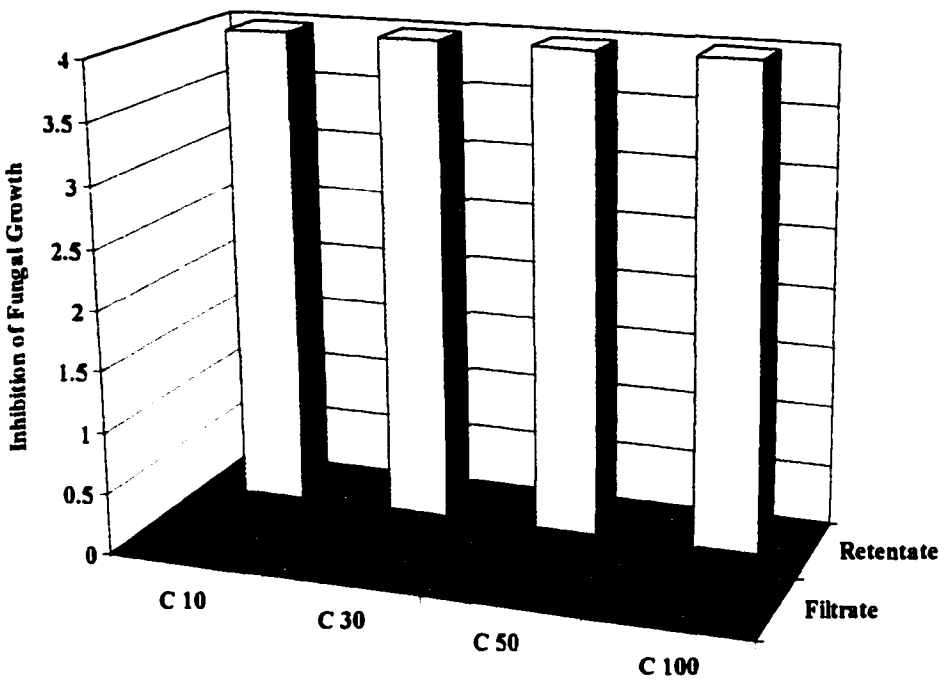


Figure VII-1 : Preliminary Size Fractionation of Antifungal Activity from Extracts of *S. cubensis* seeds. Dialyzed neutral extract was fractionated by ultrafiltration using 10, 30, 50 and 100 kDa MWCO concentrators. One hundred µl of both retentate and filtrate from each size fractionation were assayed for antifungal activity against *A. flavus*.

Ammonium sulfate fractionation

Dialyzed neutral extract from *S. cubensis* seeds was fractionated by stepwise ammonium sulfate fractionation. Pellets obtained after each ammonium sulfate fractionation were resuspended and dialyzed in 10 mM Tris-HCl, pH 8.0. The final supernatant after precipitation with 90% ammonium sulfate was dialyzed and samples were assayed for antifungal activity. Antifungal activity was present in each fraction (Figure VII-2). The best activity was in the 0-25% and 25-45% pellets. The activity was spread through all the fractions suggesting that there may be multiple activities and/or heterogeneity in the active component(s).

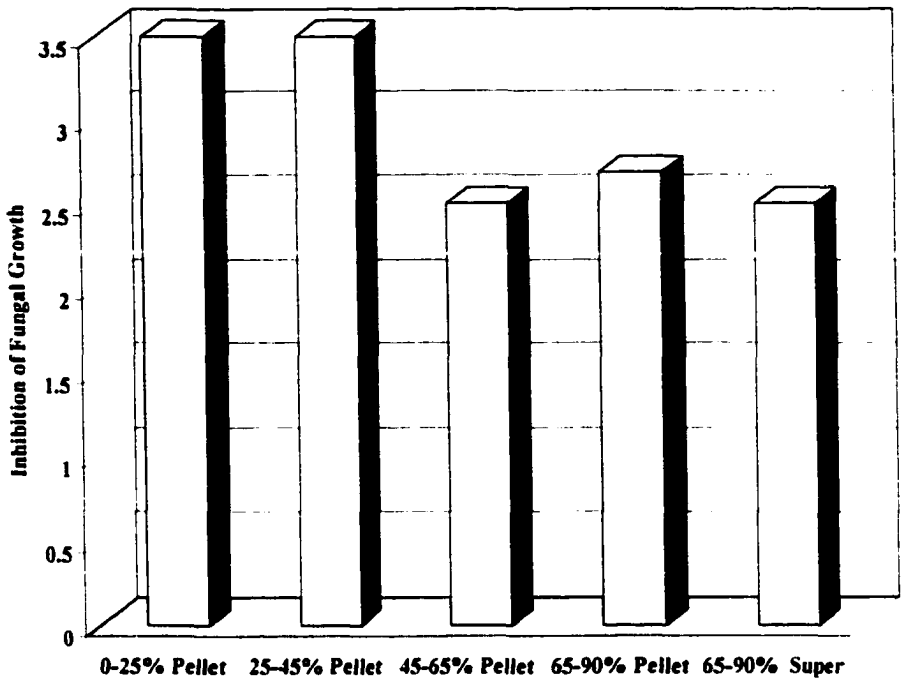


Figure VII-2: Evaluation of Antifungal Activity of Ammonium Sulfate Fractionated Neutral Extract of Seeds of *S. cubensis*. Dialyzed neutral extract was fractionated by stepwise addition of ammonium sulfate. Precipitated samples were resuspended in a minimum volume of 10 mM Tris-HCl, pH 8.0, dialyzed and assayed for antifungal activity against *A. flavus* using the standard bioassay.

PRELIMINARY PURIFICATION OF ANTIFUNGAL ACTIVITIES

Extraction Procedure

S. cubensis seeds were extracted using both neutral and acidic extraction procedures. The extraction buffer for the neutral extract was 10 mM Tris-HCl, pH 8.0 and 0.1 M HCl was used for acid extraction. Crude acid extract was either neutralized with 2 M NaOH and than dialyzed or dialyzed immediately without neutralization. Dialyzed neutral extract (NEx) and neutralized/dialyzed acid (NDAEx) extracts were assayed for antifungal activity. Dialyzed neutral extract and neutralized dialyzed acid extract completely inhibited conidial germination of *A. flavus*. Hemagglutinating activity was present in dialyzed neutral extract but was absent in dialyzed acid extract. Preliminary experiments were conducted to develop a protocol for the purification of the antifungal activities from both neutral extract and acid extract.

Size-exclusion Chromatography

Neutral extract: Dialyzed neutral extract was concentrated 2-4 fold by ultrafiltration using a Centricon C10 concentrator. The concentrated sample

(0.5 ml) was loaded onto a Superose 12 column and eluted with 10 mM Tris-HCl, pH 8.0. Protein elution from the column was measured at 280 nm (Figure VII-3). Twenty four-ml fractions were collected from the Superose 12 column and assayed for antifungal and hemagglutination activities. Strong inhibition of *A. flavus* growth was observed in fraction 10 (Figure VII-4). Moderate inhibition of hyphal growth was observed in assays containing fractions 1, 9, 11 to 13 and fractions 8 and 14 exhibited slight inhibition. Hemagglutination activity was observed in fraction 12 and 13 but not in fraction 1, 8-10, 11 or 14. Analysis of the protein-banding pattern revealed protein bands in all the fractions (Figure VII-5). The banding pattern was similar to the pattern observed in the SDS gel of *S. simplex* neutral extract Superose 12 fractions. Two major bands of approximately 31 kDa were present in fractions 4 and 5. Bands of similar size were also present in the rest of the fractions but the amount of protein was lower and they appeared as minor bands.

Acid extract: Dialyzed acid (DAEx) extract from *S. cubensis* seeds was concentrated and fractionated by size-exclusion chromatography on Superose 12. The absorbance profile (280 nm) of the eluted fractions is

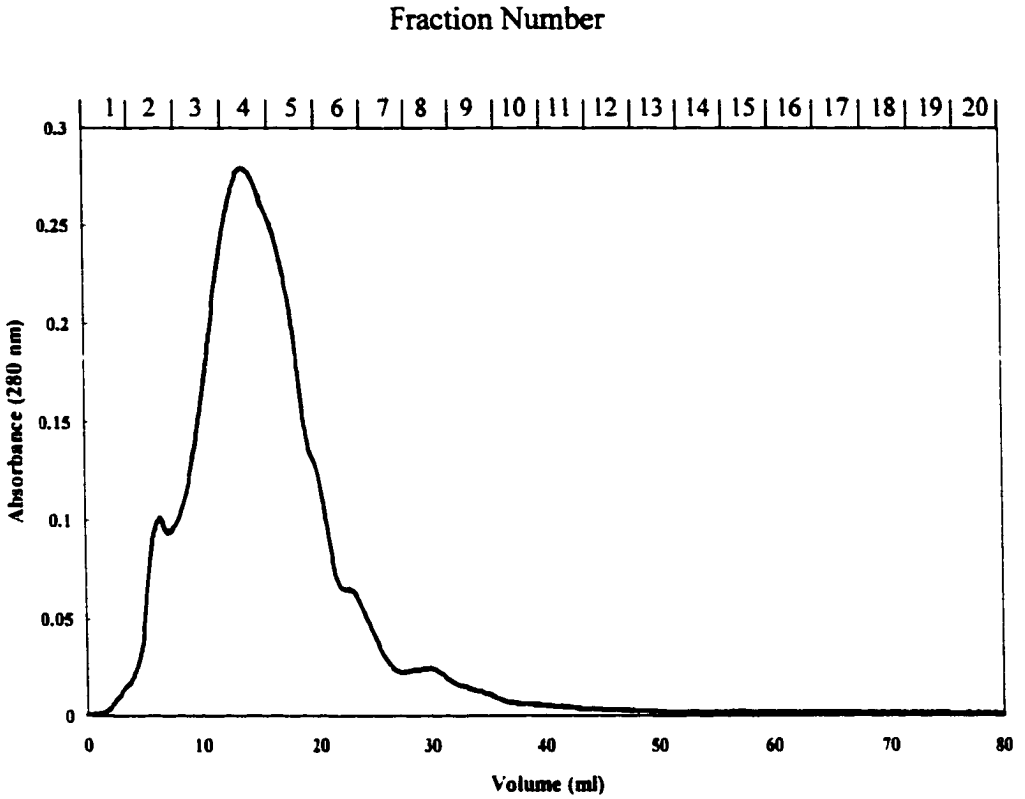


Figure VII-3 : Absorbance Profile of Neutral Extract of *S. cubensis* seeds Fractionated by Size Exclusion Chromatography. Five hundred µl of concentrated (2x) neutral extract was applied to the column. Proteins were eluted with 10 mM Tris-HCl, pH 8.0.

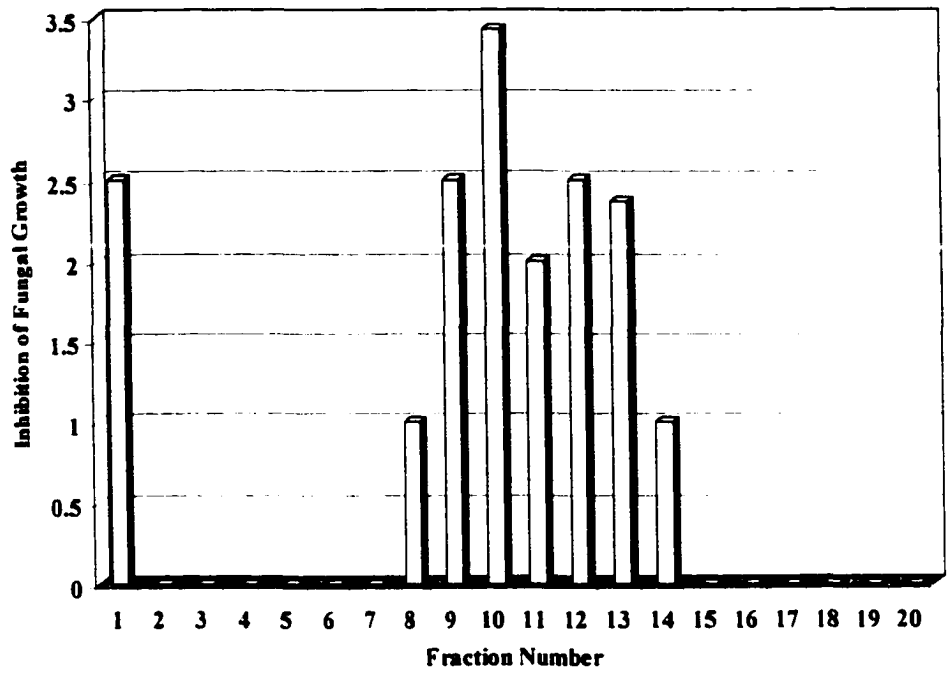


Figure VII-4 : Antifungal Activity of Fractions Obtained by Size Exclusion Chromatography of Neutral Extract of *S. cubensis* Seeds. Neutral extract was fractionated on a Superose 12 column equilibrated with 10 mM Tris-HCl, pH 8.0. One hundred µl from each fraction was assayed for antifungal activity against *A. flavus* in the standard bioassay.

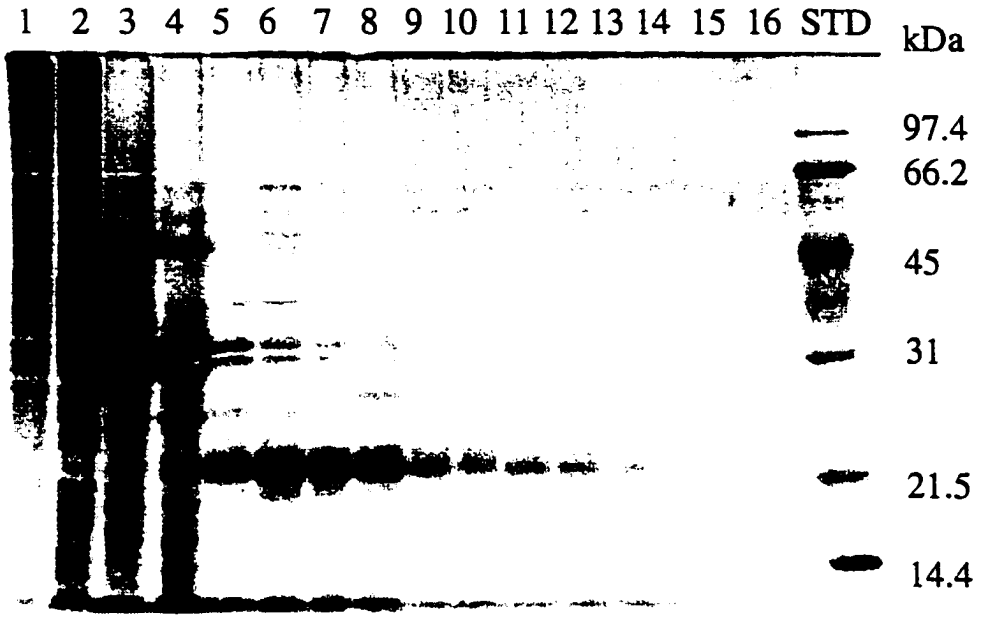


Figure VII-5 : SDS PAGE Analysis of Fractions Obtained by Size Exclusion Chromatography of Neutral Extract of S. cubensis Seeds. One hundred µl from each fraction was dried *in vacuo*, resuspended in 10 µl of SDS sample buffer and boiled for 5 min before loading onto the gel. Lane 1 to 16, Superose 12 fractions 1 to 16. STD = molecular weight standards.

shown in Figure VII-6. Fractions obtained after size-exclusion chromatography of the acid extract were assayed for antifungal activity. Although the acid extract completely inhibited fungal growth, none of the fractions from the Superose 12 column inhibited growth of *A. flavus*. The protein composition of each of the fractions was examined by SDS PAGE (gel not shown). Protein bands were detected in fractions 2 to 12 but not in any of the other fractions even though, materials were eluted with absorbance at 280 nm. According to the SDS gel profile and antifungal assay, size-exclusion chromatography on Superose 12 was not an effective method for the preliminary purification of antifungal activities from acid extract of *S. cubensis* seeds.

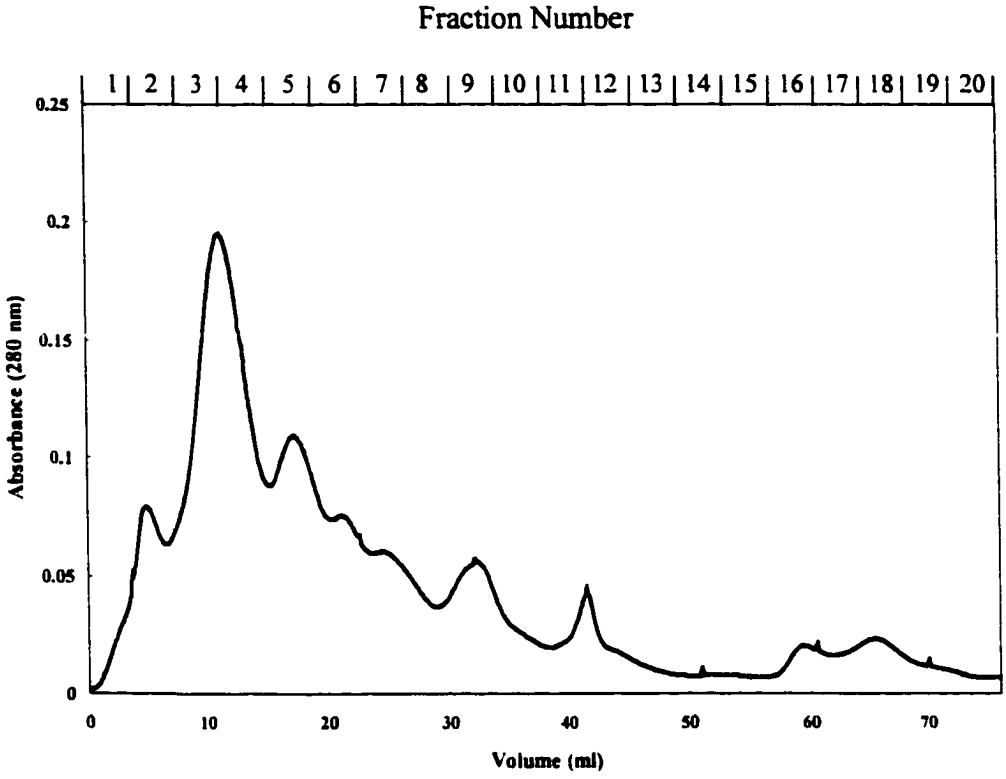


Figure VII-6 : Absorbance Profile of Size Exclusion Chromatography of Acid Extract of *S. cubensis* Seeds. Five hundred µl of concentrated (2x) acid extract was loaded onto the column. Proteins were eluted with 10 mM Tris-HCl, pH 8.0. Twenty 4-ml fractions were collected.

Affinity chromatography

Chitin-affinity chromatography was a more effective method of preliminary purification of the antifungal activities present in both acid and neutral extracts of *S. cubensis* seeds. Figure VII-7 summarizes the methods used for purification of antifungal activities by chitin-affinity chromatography along with a summary of key results.

Neutral chitin column: Two different protocols for chitin-affinity chromatography of neutral extract were used. In both cases, 10 ml of dialyzed neutral extract was applied to the column. The flow through was collected and reapplied to the column 4 times. Proteins not bound to the column were washed from the column with 100 ml of 10 mM Tris-HCl, pH 8.0. At this point, the two procedures diverge. Bound proteins were eluted with either low and high pH or a step gradient of NaCl (i. e. 100 ml of 0.1 M, 0.2 M, 0.3 M, 0.4 M, 0.5 M and 1M NaCl in 10 mM Tris-HCl, pH 8.0) according to the methods described in Chapter IV.

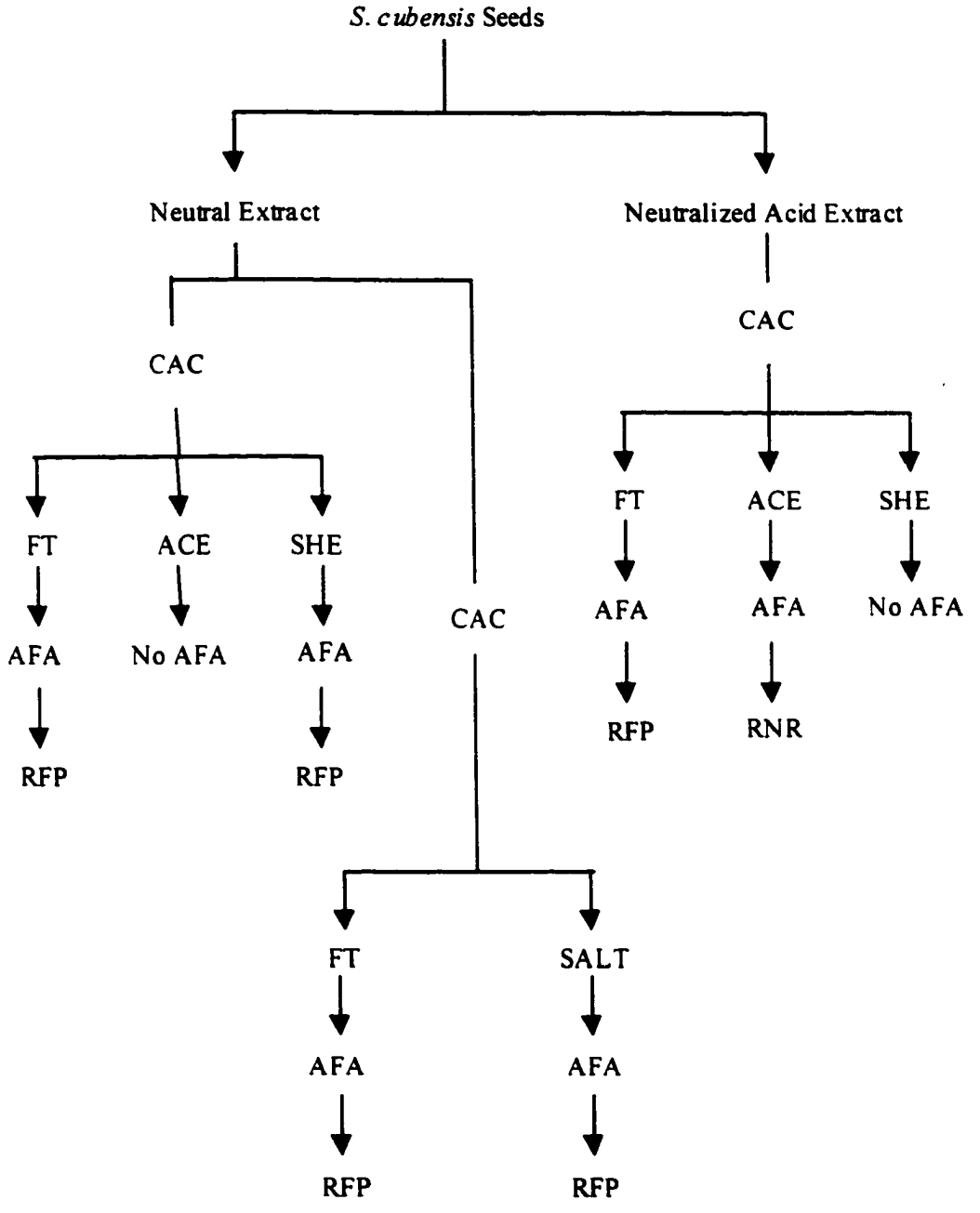


Figure VII-7: Summary of Results from Chitin Affinity Chromatography of Extracts of *S. cubensis* Seeds.

Abbreviations: CAC, chitin affinity chromatography; FT, flow through; ACE, acetic acid eluate; SHE, sodium hydroxide eluate; SALT, fractions eluted with salt; AFA, antifungal activity; No AFA, no antifungal activity; RFP; requires further purification; RNR, results not reported herein.

Using the first method, chitin-binding proteins were eluted with 0.1 M acetic acid followed by 0.1 M NaOH. The acetic acid eluate exhibited very little absorbance at 280 nm and after ammonium sulfate precipitation of this fraction there was no visible pellet. On the other hand, fractions eluted with sodium hydroxide yielded a sizable pellet that was resuspended in 4 ml of 10 mM Tris-HCl, pH 8.0. Except the flow through and first Tris-HCl wash, all the other fractions were dialyzed against 10 mM Tris-HCl, pH 8.0 and all fractions were assayed for antifungal activity. Antifungal activity was present in the flow through, first Tris wash, 0.1 M salt wash and sodium hydroxide eluate (Figure VII-8). The sodium hydroxide eluate completely inhibited conidial germination and the flow through strongly inhibited hyphal growth. The first Tris wash and the 0.1 M salt wash exhibited only moderate inhibition of fungal growth.

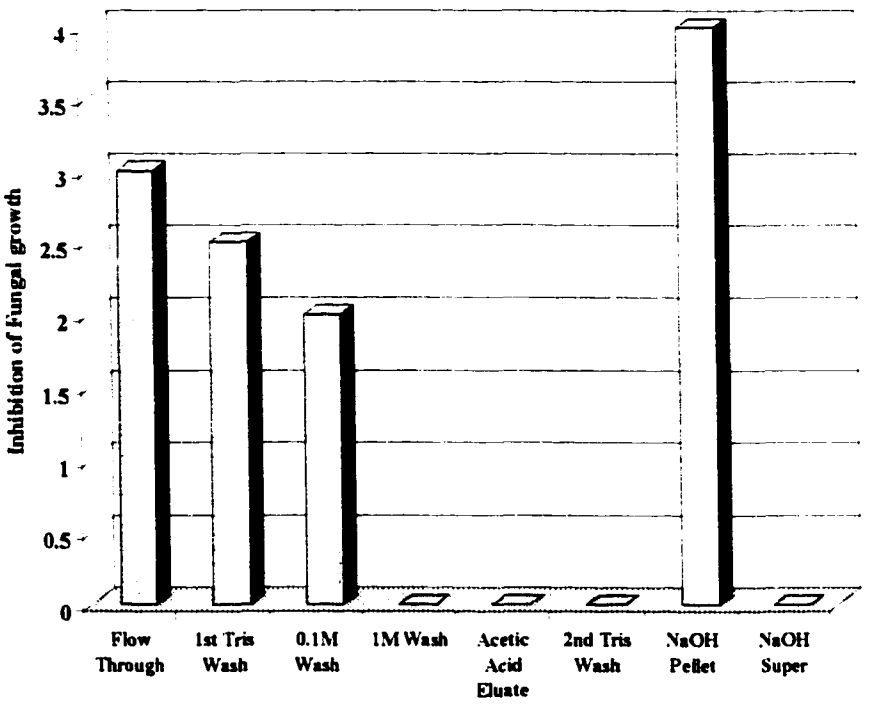


Figure VII-8: Antifungal Activity of Fractions from Neutral Chitin Column. Ten ml of dialyzed neutral extract of *S. cubensis* seeds was fractionated on a chitin affinity column. Fractions from the chitin column were dialyzed against 10 mM Tris-HCl, pH 8.0, and assayed for antifungal activity using the standard bioassay with *A. flavus*.

Protein bands were visible in all fractions on the SDS gel (Figure VII-9). In the flow through, bands with estimated molecular weights of 21-24 kDa were very prominent. Several additional bands were visible in the flow through, first Tris-HCl wash and 0.1 M salt wash. The intensity of staining of all of these bands was lower in the first Tris-HCl wash and 0.1 M salt wash. There was no visible protein band in the 1 M salt wash and the supernatant of the sodium hydroxide eluate after ammonium sulfate precipitation. The acetic acid eluate contained only one protein band of 31 kDa. The sodium hydroxide eluate also contained one 31-kDa band and two bands with molecular weights smaller than 21 kDa

The chitin column fractions eluted with salt were dialyzed and assayed for antifungal activity. According to the results of the bioassay, the flow through, 0.1 M and 0.4 M salt eluted fractions showed very strong inhibition of fungal growth while the 0.5 M and 1 M salt eluted fractions completely inhibited conidial germination (data not shown). SDS-PAGE analysis of the salt eluted chitin column fractions revealed several protein bands in all the fractions but the number of bands was higher in the flow through and 0.1 M salt eluted fractions (gel not shown). Even though both

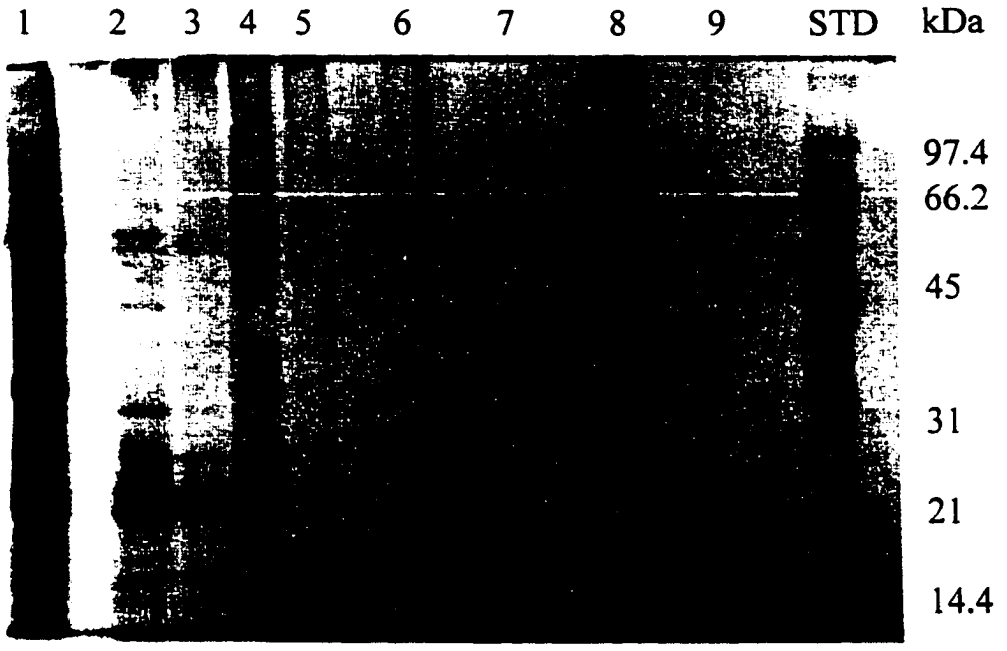


Figure VII-9: SDS-PAGE Gel of *S. cubensis* Neutral Chitin Column Fractions. One hundred µl of each fraction was dried *in vacuo*, resuspended in 10 µl of SDS sample buffer and boiled for 5 min before loading onto the 12.5% SDS gel. Lane 1, neutral extract; lane 2, flow through; lane 3, 1st Tris wash; lane 4, 0.1 M salt wash; lane 5, 1 M salt wash; lane 6, acetic acid eluate; lane 7, 2nd Tris wash; lane 8, sodium hydroxide eluate; lane 9, sodium hydroxide supernatant. Protein bands were revealed by silver staining. STD = molecular weight standards.

procedures were effective, efforts focused on the use of low and high pH elution for the continued purification of antifungal activity from extracts of *S. cubensis* seeds.

Chitin-affinity chromatography of acid extract: *S. cubensis* seeds were extracted in 0.1 N HCl according to the method described in Chapter IV. Seeds (10 g) were extracted in 50 ml of 0.1 N HCl and centrifuged at 20,000 x g for 10 min. After centrifugation, the pellet was discarded and the pH of supernatant fluid was adjusted to pH 3.8 with 2 N NaOH and allowed to stand on ice for 1 h. After 1 h, the supernatant was centrifuged again at 20,000 x g for 10 min. The clear supernatant fluid after centrifugation was filtered through Whatman 3 MM filter paper and five ml of the filtrate was loaded onto a chitin-affinity column equilibrated with 50 mM sodium acetate buffer, pH 3.8. The remainder (approximately 10 ml) of the neutralized acid extract was dialyzed against 10 mM Tris-HCl, pH 8.0. Unbound proteins were washed from the column with 100 ml of 0.1 M NaCl followed by 100 ml of 1 M NaCl both in 50 mM sodium acetate, pH 3.8. After removal of the proteins which did not bind or bind tightly to the chitin column, bound proteins were eluted first with 0.1 N acetic acid and then with 0.1 N NaOH according to the procedure described in the Materials and

Methods of Chapter IV. Between the high and low pH elution the column was washed with 1 M NaCl in 50 mM sodium acetate, pH 3.8. Fractions were dialyzed and/or concentrated as noted.

Strong antifungal activity was observed in the flow through, acetic acid eluate (Figure VII-10). Moderate inhibition was present in the acetic acid supernatant indicating that not all of the antifungal activity was precipitated with ammonium sulfate. No antifungal activity was observed in the NaOH eluate. In contrast to results with *S. simplex*, the antifungal activity in the extract of *S. cubensis* seeds was not denatured by acid extraction and neutralization. Strong inhibition of *A. flavus* hyphal growth was observed in the neutralized dialyzed acid extract.

Many of the proteins present in the extracts, however, were denatured by this process. Analysis of neutralized dialyzed acid extract on SDS-PAGE revealed only five protein bands (Figure VII-11). These same protein bands were also observed in the chitin column flow through although the intensity of the bands was reduced in the flow through. The resuspended and dialyzed acetic acid eluate after ammonium sulfate precipitation showed several very prominent bands with molecular weights less than 21 kDa.

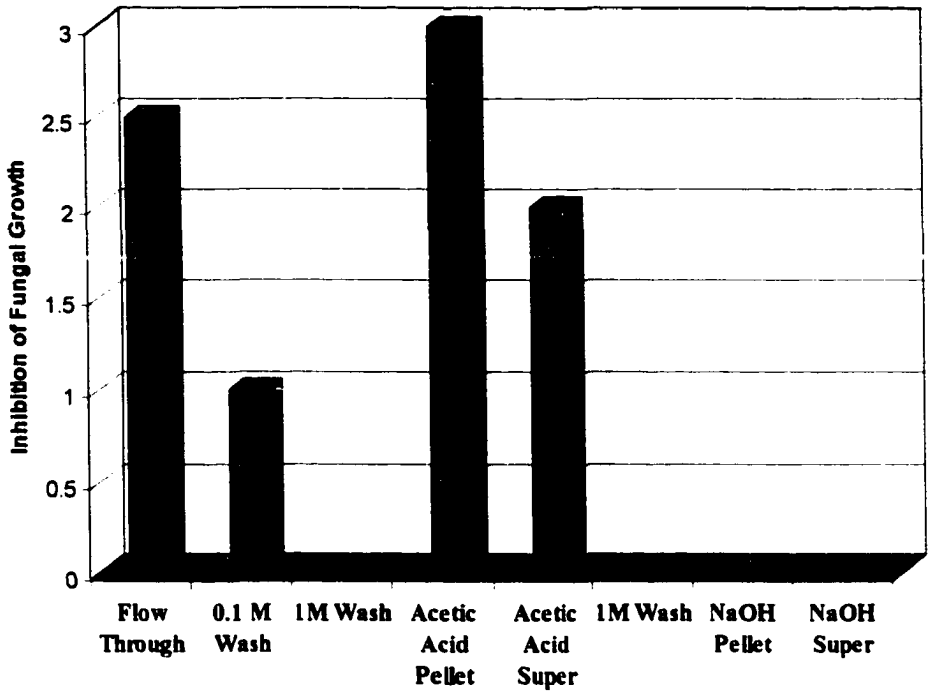


Figure VII-10: Antifungal Activity of *S. cubensis* Acid Chitin Column Fractions. Seeds were extracted in 0.1 M HCl and the extract was neutralized with NaOH. Ten ml of the neutralized acid extract was applied to a chitin-affinity column. The fractions from the chitin column were dialyzed in 10 mM Tris-HCl, pH 8.0, and assayed for antifungal activity against *A. flavus*.

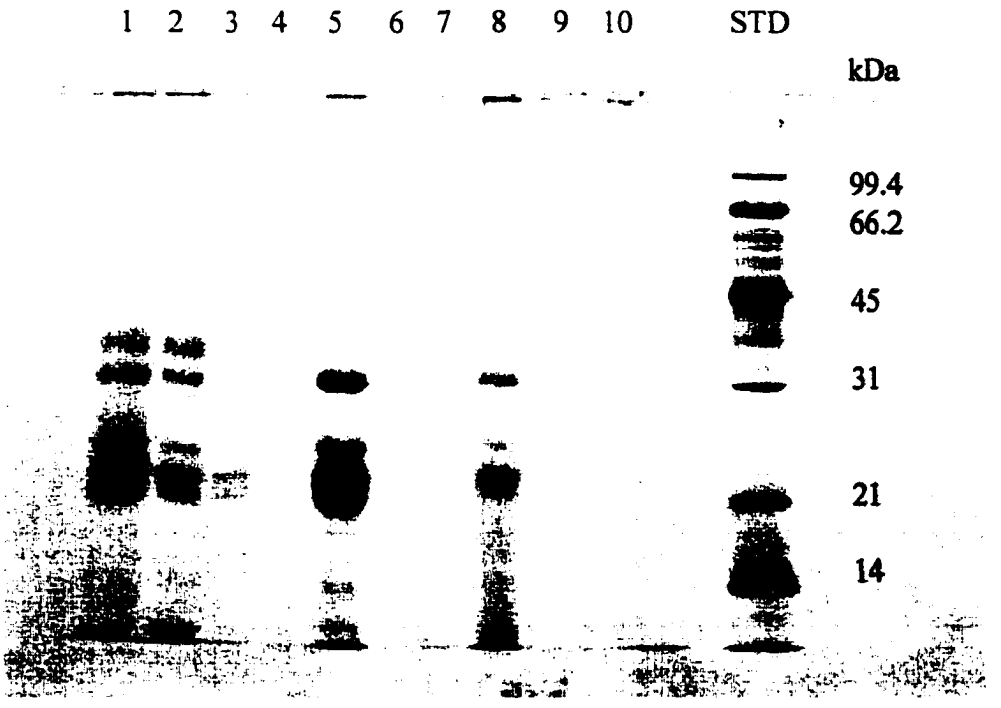


Figure VII-11: SDS-PAGE Gel of *S. cubensis* Acid Chitin Column Fractions. One hundred µl of each fraction was dried *in vacuo*, resuspended in 10 µl of SDS sample buffer and boiled for 5 min before loading onto the 12.5% SDS gel. Lane 1, neutralized/dialyzed acid extract (20 µl dried); lane 2, flow through; lane 3, 0.1 M salt wash; lane 4, 1 M salt wash; lane 5, acetic acid eluate; lane 6, acetic acid supernatant; lane 7, 2nd 1 M salt wash; lane 8, sodium hydroxide eluate; lane 9, sodium hydroxide supernatant; Gel was silver stained to reveal protein bands. STD = molecular weight standards.

Although much less abundant, bands of similar size were also observed in the sodium hydroxide eluate. A 31-kDa band was also present in acetic acid eluate, sodium hydroxide eluate and in the flow through.

Other Methods

Anion-exchange chromatography: Anion-exchange chromatography was initially evaluated as a method for the preliminary purification of antifungal activity from extracts of *S. cubensis* seeds. Dialyzed neutral extract was fractionated on a Pharmacia RSQ column according to the procedure described in the Materials and Methods (Chapter IV). Fractions obtained after anion-exchange chromatography were dialyzed (10 mM Tris-HCl, pH 8.0) and assayed for antifungal activity. Activity was present in every fraction indicating either heterogeneity of the active species or the presence of more than one activity (data not shown). Although agglutination activity was detected in the dialyzed neutral extract, there was no agglutination in the RSQ fractions. An SDS gel of the RSQ fractions (gel not shown) revealed a similar pattern of protein bands (21 to 45 kDa) as observed in the preliminary anion-exchange fractionation of dialyzed neutral extract of *S.*

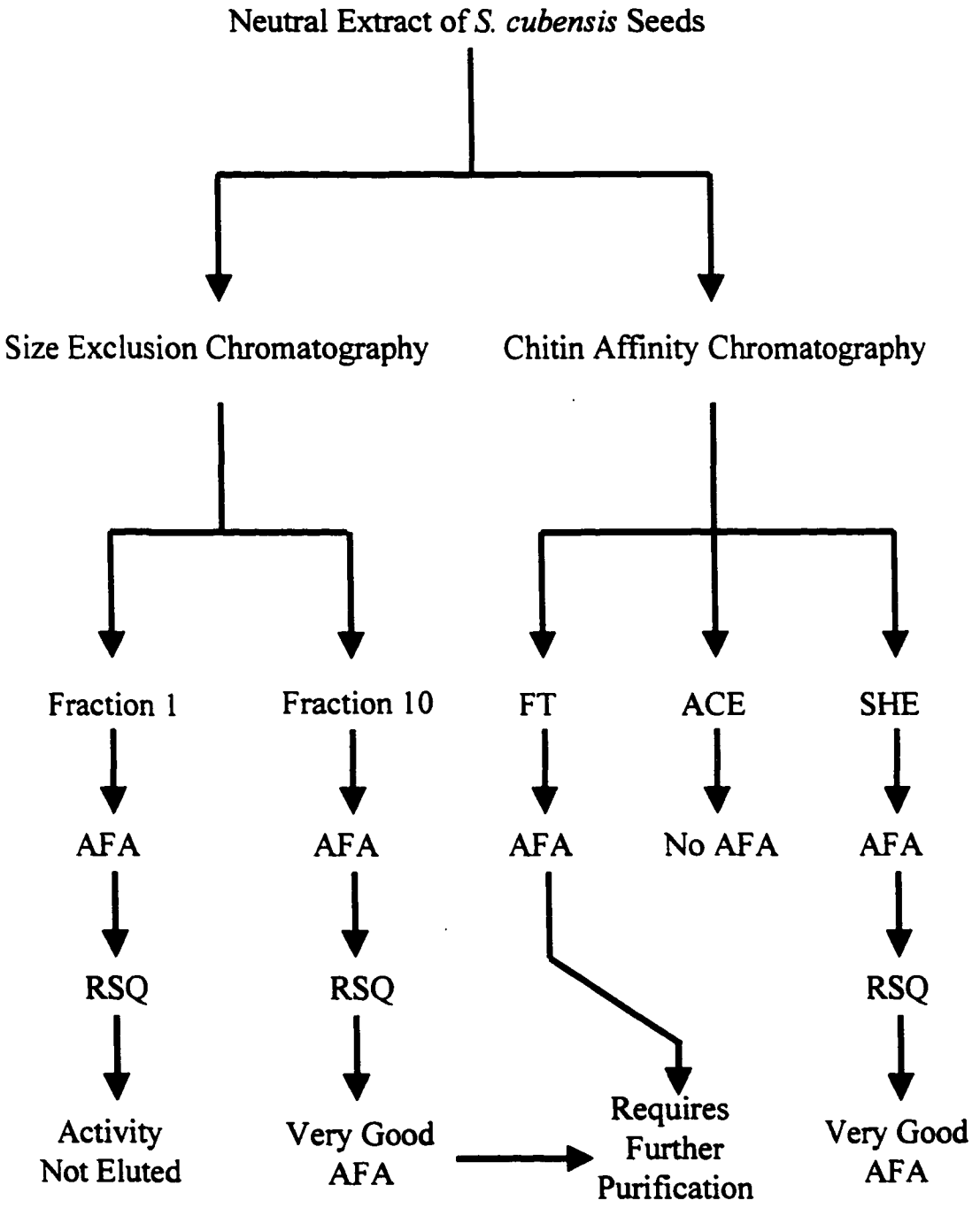
simplex seeds. Antifungal activity was not resolved well by anion-exchange chromatography when used as a preliminary step of purification.

Preparative isoelectric focusing: Dialyzed neutral extract (35 ml) was fractionated by preparative isoelectric focusing according to the procedures described in the Materials and Methods section of Chapter IV. None of the 20 fractions possessed antifungal activity suggesting that the activity did not survive the separation process.

PURIFICATION OF ANTIFUNGAL ACTIVITY

Two protocols for the purification of antifungal proteins from *S. cubensis* are presented herein. Both protocols used dialyzed neutral extracts of *S. cubensis* seeds as the starting material. Although neutralized acid extract contained very good antifungal activity, efforts to purify these components are not presented. The two protocols used for further purification of antifungal activity along with a summary of key results are presented in Figure VII-12.

Figure VII-12: Summary of Final Purification Protocols for the Isolation of Antifungal Proteins from Extracts of *S. cubensis*.



Anion-exchange chromatography of neutral extract Superose 12 fractions: According to the results of the fungal bioassay, fraction 10 from the neutral extract separated on Superose 12 exhibited strong antifungal activity. Moderate activity was observed in fractions 1, 9 and 11 to 13. Selected active fractions were pooled and further fractionated by anion-exchange chromatography.

Fraction 1 was pooled from three different Superose 12 runs and 10 ml of the pooled fraction was applied to a Pharmacia RSQ column. Proteins were eluted with a gradient of 0 to 1 M NaCl. Fractions were collected, dialyzed and assayed for antifungal activity. Based on the fungal assay, there was no inhibition of *A. flavus* growth in any of these fractions. Likewise, there were no protein bands detected on the SDS gel of these fractions. These results were completely opposite to those obtained with anion-exchange fractions of *S. simplex* neutral extract Superose 12 fraction 1.

Fraction 10 from the neutral extract Superose 12 run were pooled and 10 ml of the pooled fraction was subjected to anion-exchange chromatography on a RSQ column. Fractions were collected, dialyzed and

concentrated for the antifungal assay. Fraction 3 to 6 completely inhibited conidial germination and fraction 7 and 9 exhibited strong inhibition of fungal growth (Figure VII-13). Analysis of a SDS gel of these fractions revealed several protein bands between 66 and 14 kDa (data not shown). Size-exclusion chromatography coupled with anion-exchange chromatography were insufficient to separate and identify the active components. Therefore, further purification using alternative methods was required.

Anion-exchange chromatography of neutral extract chitin column fractions: According to the results of the antifungal assay of neutral extract chitin column fractions, antifungal activity was present in the flow through, first Tris-HCl wash and sodium hydroxide eluate. The latter completely inhibited conidial germination of *A. flavus*. On this basis, antifungal activity from the sodium hydroxide eluate was further purified by anion-exchange chromatography on a Pharmacia RSQ column. The sodium hydroxide pellet was loaded onto the RSQ column and proteins were eluted as stated in the Materials and Methods (Chapter IV). Eleven fractions (Figure VII-14) were collected, dialyzed and assayed for antifungal activity. Results of the

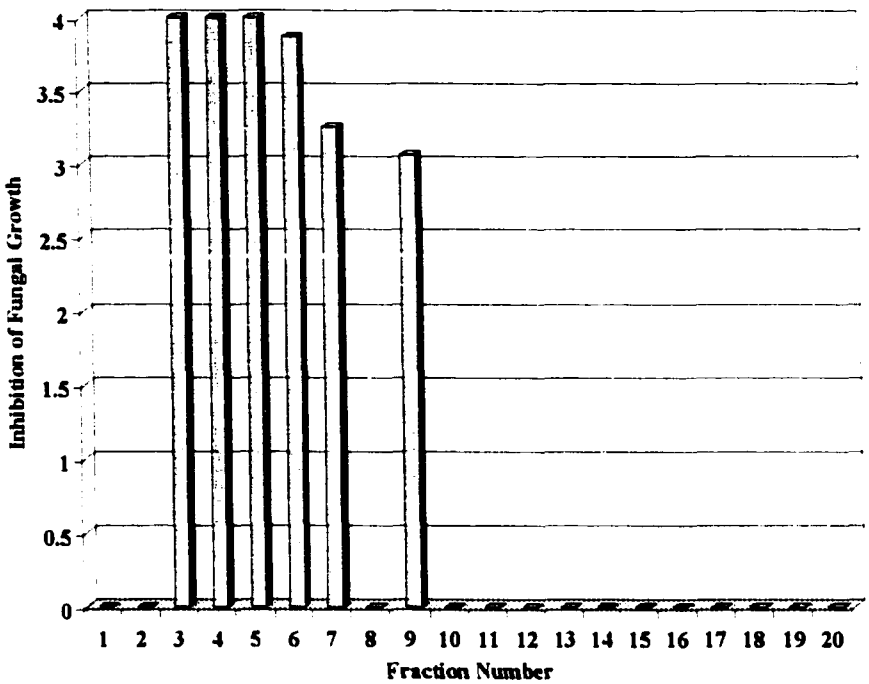


Figure VII-13: Antifungal Activity of Fractions after Anion Exchange Chromatography of Superose 12 Fractions 9 and 10. Fraction 9 and 10 were obtained from size fractionation of *S. cubensis* neutral extract on Superose 12. Both of these fractions were pooled and 10 ml of the pooled material was separated on a RSQ column. Proteins bound to the column were eluted with a linear gradient of NaCl. All the fractions from the column were dialyzed against 10 mM Tris-HCl, pH 8.0, and assayed for antifungal activity against *A. flavus*.

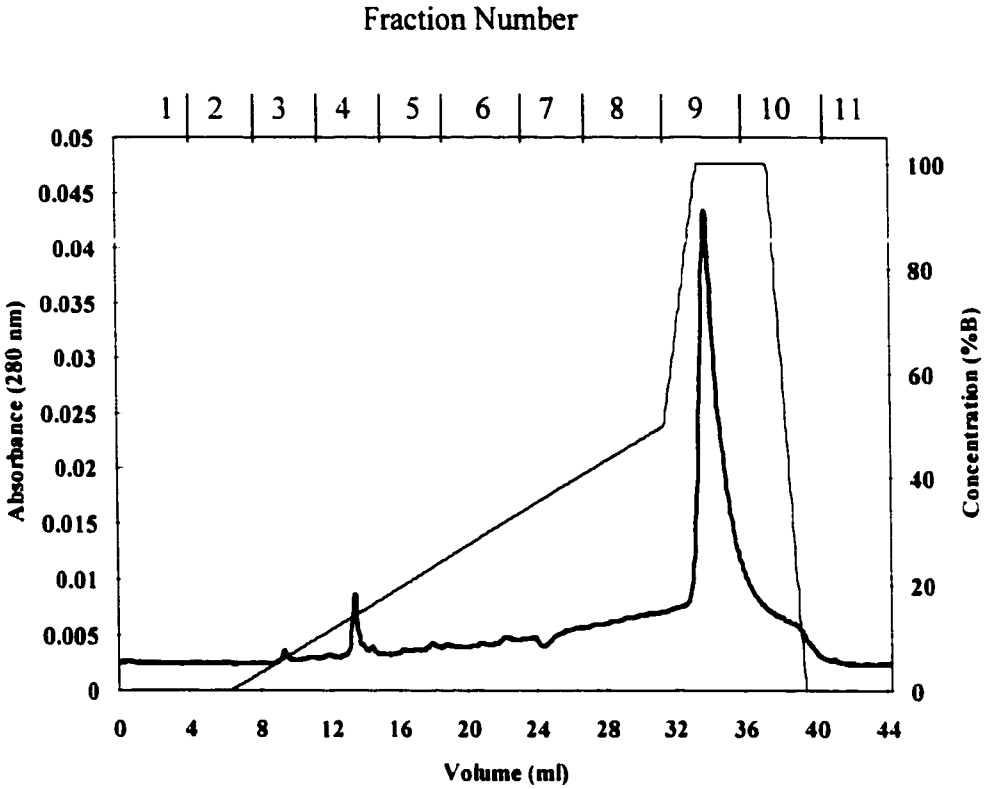


Figure VII-14: Absorbance Profile of Anion Exchange Column Chromatography of the Sodium Hydroxide Eluate from the *S. cubensis* Neutral Chitin Column. Five hundred µl of the sodium hydroxide eluate was applied to the column. Bound proteins were eluted with a linear gradient of sodium chloride in 10 mM Tris-HCl, pH 8.0.

antifungal assay after incubation for 48 h indicated that antifungal activity was present in all the fractions except fraction three and eleven (Figure VII-15). Moderate activity was present in fractions 4 to 7 while strong antifungal activity was present in fraction 1. Fractions 8-10 exhibited very strong to complete inhibition of conidial germination. Observations of these assays after prolonged incubation (three weeks) revealed moderate inhibition in fractions 4 while fraction 9 still completely inhibited fungal growth.

SDS-PAGE analysis revealed a 31 to 33-kDa protein band in fractions 4, 5 and 6 but the intensity of this band was lower in fraction 6 (Figure VII-16). These bands were similar in size to chitinases from *S. simplex* seeds. A number of lightly-stained protein bands were visible in fractions 7 to 10. Although it is possible that the antifungal activity in these fractions could be due to one or more of these proteins, it is not possible from these results to identify with any certainty the antifungal compound. The possibility exists that the some other compound in these fractions and not a protein is responsible for the activity. Fractions 1 to 3 each had a protein band of molecular weight close to 41 kDa. The antifungal activity decreased with the decreasing intensity of staining of this band.

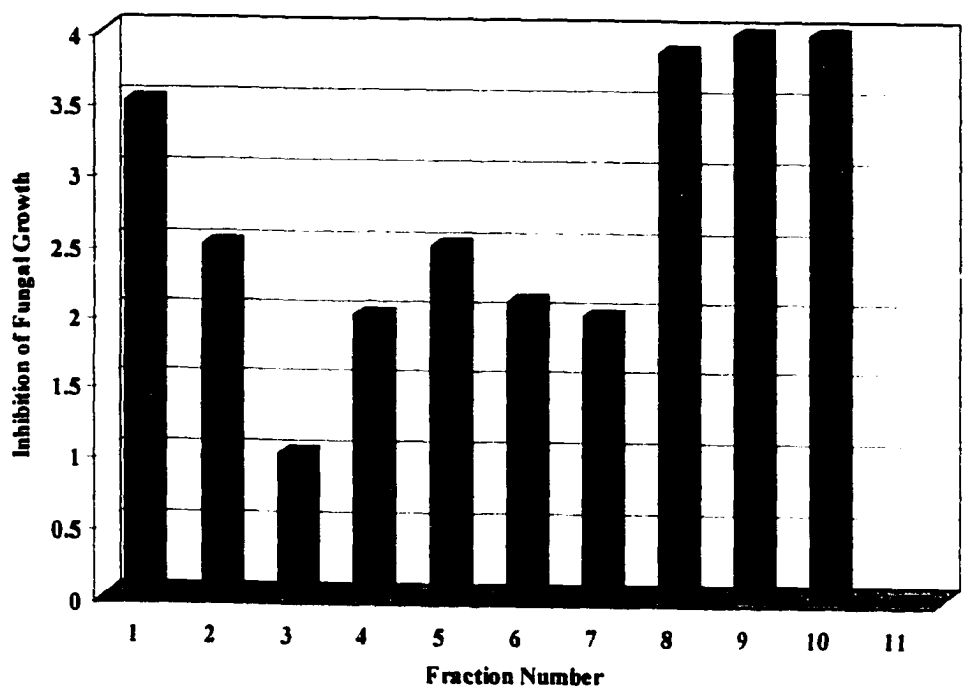


Figure VII-15: Antifungal Activity of Fractions Obtained After Anion-Exchange Chromatography of Sodium Hydroxide Eluate from the *S. cubensis* Neutral Chitin Column. Sodium hydroxide eluate from the neutral chitin column was fractionated on a Pharmacia RSQ column. All the fractions were dialyzed against 10 mM Tris-HCl, pH 8.0, and 100 µl of each fraction was assayed for antifungal activity against *A. flavus*.

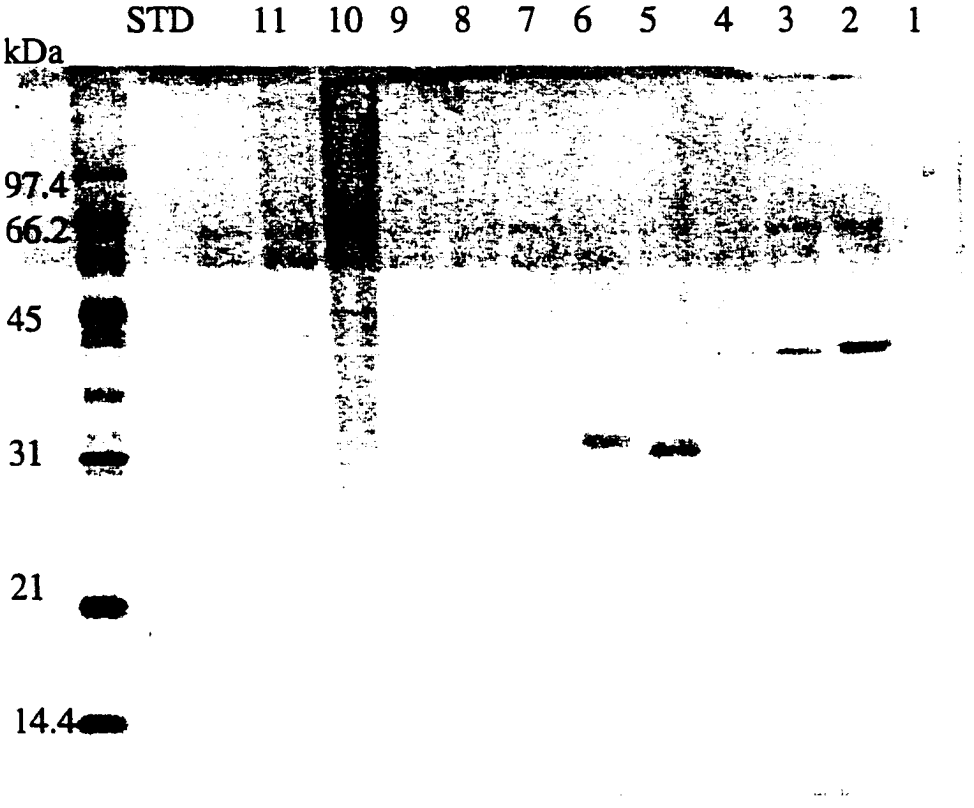


Figure VII-16: SDS-PAGE Analysis of Fractions from the Anion Exchange Column Fractionation of the Sodium Hydroxide Eluate. Sodium hydroxide eluate from the *S. cubensis* neutral chitin column was applied to a RSQ column. Two-hundred µl samples from each fraction were dried *in vacuo*, resuspended in 10 µl of SDS sample buffer and boiled for 5 min before loading onto the 12.5% SDS gel. Lane 1 to 11, RSQ fractions (see Figure VII-14) 1 to 11. The gel was silver stained to reveal protein bands. STD = molecular weight standards.

CHARACTERIZATION

Selected chitinases are apparently responsible, at least in part, for the antifungal activity present in extracts of *S. simplex* seeds. These chitinases had molecular weights in the range of 28-36 kDa and were purified by chitin-affinity chromatography coupled with anion-exchange chromatography. Using the same basic purification protocol, protein bands of similar size were detected in anion-exchange column fractions exhibiting moderate antifungal activity and obtained from extracts of *S. cubensis* seeds. The moderate level of activity may reflect the very low levels of proteins of these fractions. To determine whether chitinase activity was present in these and other fractions and might be responsible, at least partially, for the antifungal activity, samples from each of the RSQ column fractions were tested for chitinase activity using the in-gel assay.

In extracts of *S. cubensis* seeds, six chitinase bands were observed in the dialyzed neutral extract (Figure VII-17). After the neutral extract was fractionated by affinity chromatography, chitinase was only detected in the sodium hydroxide eluate (five chitinase bands). After purification of the sodium hydroxide eluate by anion-exchange chromatography, chitinase

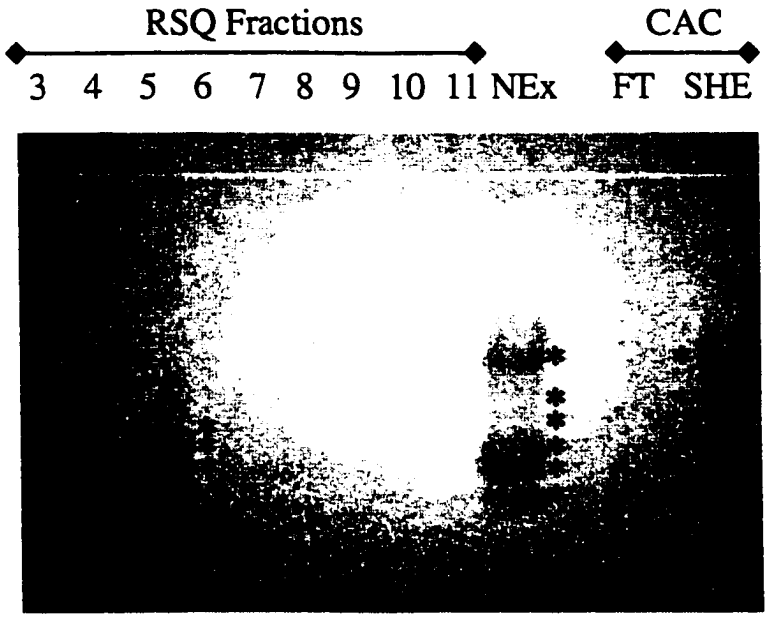


Figure VII-17: Detection of Isoforms of Chitinases in Selected Fractions of *S. cubensis* Seed Extract. Neutral extract of *S. cubensis* seeds was fractionated on a chitin column and the sodium hydroxide eluate from the neutral chitin column was further fractionated by anion exchange chromatography. Ten µl of selected chitin column fractions was separated on a 12.5% native gel. Sodium hydroxide eluate from the chitin column was fractionated on a RSQ column. A sample from each fraction was concentrated and 10 µl of each of the concentrated sample was separated on the same gel along with the chitin column fractions. Chitinase activity was detected using an in-gel assay. Samples: RSQ fractions 3 to fraction 11; neutral extract (NEx); flow through (FT); and sodium hydroxide eluate (SHE), respectively. Chitinase bands are indicated with (*).

bands were detected in fractions 4 and 5 (Figure VII-17). Two chitinase bands were detected in fraction 4 (chitinase B1, B2) and three chitinase bands were detected in fraction 5 (chitinase B3, B4 and B5). The sixth form of chitinase present in the original extract was not isolated or not detected under these conditions.

Fractions 4 and 5 exhibited moderate inhibition of *A. flavus* hyphal growth (Figure VII-15) in the 48 h assay but activity in fraction 5 decreased with prolonged incubation. After 4 weeks of incubation, inhibition of *A. flavus* hyphal growth by fraction 5 was overcome. Fraction 4, on the other hand still, inhibited hyphal growth. In the initial 48-h assay, fractions 8, 9 and 10 completely inhibited fungal growth. Even after 4 weeks of incubation, fraction 9 still completely inhibited growth of *A. flavus*. This chitin-binding activity did not correspond with chitinase activity.

ANALYSIS AND DISCUSSION OF RESULTS

To date, there has been no report on the occurrence of antifungal proteins in *S. cubensis*. As far as known, this is the first report on the presence and isolation of antifungal proteins including chitinases and other antifungal proteins from *S. cubensis* seeds.

Purification of antifungal activities from the extracts of *S. cubensis* seeds was carried out in parallel with the purification of active proteins from *S. simplex*. Because both of these species belong to the same genus, one might assume that the antifungal activities in the seeds of these two species would be quite similar. Although there were some similarities between antifungal activities present in the two species, a number of differences were also apparent from the earliest stages of the purification.

In both the species, there appear to be multiple activities. The properties of the antifungal activities suggest that the active factors are proteins. Ammonium sulfate precipitation and size fractionation along with the heat treatment gave a clear indication of the proteinaceous nature of the activity. Antifungal activities in *S. cubensis* seeds were denatured by a 50°C heat treatment and were greater than 3.5 kDa in molecular weight. Antifungal activity was precipitated over a wide range of ammonium sulfate

concentrations providing evidence for the existence of multiple antifungal proteins. In *S. simplex*, agglutinating activity was correlated with one form of antifungal activity. Agglutinating activity was sensitive to low pH. Low pH caused the loss of antifungal and agglutinating activity in Superose 12 fractions 12 and 13, the peak of agglutinating activity in the neutral extract of *S. cubensis* seeds. The corresponding loss of both antifungal activity and agglutination adds support to the hypothesis that the two are connected.

At least some of the agglutinin was heat stable and was not precipitated with ammonium sulfate. In extracts of *S. cubensis* seeds, agglutinating activity present in the neutral extract was not denatured by heat treatment up to 80°C but was denatured by the 90°C heat treatment. The loss of antifungal activity after the 50°C treatment, argues against the notion that the agglutinating activity (at least the heat-resistant agglutinating activity) was responsible for the antifungal activity.

Four different preliminary purification procedures were evaluated. These were size-exclusion-chromatography, chitin-affinity chromatography, anion-exchange chromatography and preparative isoelectric focusing. The procedures mentioned above were chosen to separate antifungal activities by utilizing different properties of proteins such as size, affinity for other

biological compounds, charge etc. Preparative IEF proved to be an unsatisfactory method for the preliminary separation because activity did not survive this procedure. Anion-exchange chromatography of neutral extract did not adequately separate antifungal activity. This may have been due to the heterogeneity of the activity or the presence of more than one activity. This phenomenon was also observed in the separation of *S. simplex* neutral extract by anion-exchange chromatography.

In contrast to results with extracts of *S. simplex* seeds, only two zones of antifungal activity were observed after separation of neutral extract on Superose 12. Antifungal activity was not recovered after further fractionation of Superose 12 fraction 1 by anion-exchange chromatography. Activity in this fraction was not eluted and remained tightly bound to the RSQ column or activity was lost during this step. In fact, no protein bands were detected on a SDS gel of the anion-exchange fractions even though protein bands were present in the starting material (fraction 1). This observation confirms the conclusion that the antifungal protein remained tightly bound to the column.

The second zone of activity occurred in Superose 12 fractions 8 to 14, Activity in these fractions was eluted as a broad peak. In contrast with

results from the purification of proteins from *S. simplex*, antifungal activities in fractions 9 and 10 were not associated with hemagglutination activity. Hemagglutinating activity, however, was present in fractions 12 and 13. Both of these fractions exhibited moderate antifungal activity.

Although some of the antifungal activity from the neutral extract was bound to the chitin column, a large portion of the activity did not bind to the column and came through in the flow through. A similar percentage of the total activity came out in the flow through when smaller volume of extract was applied to the column, suggesting that the column was not overloaded. During the purification of antifungal activities from *S. simplex* by chitin-affinity chromatography, antifungal activity was eluted by both acetic acid and sodium hydroxide. With neutral extracts of *S. cubensis* seeds, the bound activity was eluted only with high pH. Six isomers of chitinase were observed in neutral crude extract of *S. cubensis* seeds. Five out of the six were present in the sodium hydroxide eluate. It is worth mentioning that chitinase activity was not detected in the flow through, suggesting that all of the *S. cubensis* chitinases contain a chitin-binding domain. When sodium hydroxide eluate from the neutral chitin column was further fractionated on an anion-exchange column, all fractions except fractions 3 and 11 caused moderate to complete inhibition of fungal growth.

Overall, there were fewer isoforms of chitinase in seeds of *S. cubensis*. Chitinases were detected in fractions 4 and 5, both of which exhibited moderate antifungal activity. Three different isoforms of chitinase were present in fraction 5 and two in fraction 4. SDS-PAGE analysis of these fractions revealed a single band with estimated molecular weight of 31 and 32 kDa in fraction 4 and 5, respectively. Amino acid sequence analysis of these proteins will reveal more information about the exact nature of these chitinases. Chitinases were not detected in any other fraction from the anion-exchange column.

SDS-PAGE analysis of fractions 1 to 3 revealed a single 41-kDa band in each fraction. No other bands were visible even after silver staining the gel. The intensity of staining of the 41-kDa band was highest in fraction 1 which exhibited the strongest antifungal activity (rating 3.5) among these three fractions. The intensity of this band gradually decreased, as did the antifungal activity with fraction 3 showing very little antifungal activity (rating 1). It is quite possible that the 41-kDa band in fraction 1 to 3 is responsible for the antifungal activity. Hemagglutination activity of the anion-exchange column fractions was not determined. The possibility that the antifungal activity in fraction 1 to 3 could be an agglutinin can not be

excluded. Antifungal activity in the rest of the anion-exchange column fractions could not be associated with any specific protein band because there were a number of faint protein bands observed on SDS gels of these fractions. No single band seemed to correspond to the activity in these fractions.

Glucanase activity was detected in the crude neutral extract (Data not shown). It is possible that some of the antifungal activity, especially activity in the neutral chitin column flow through could be due to glucanases. Protein bands close to the molecular weight of glucanases were observed after SDS-PAGE analysis of the neutral chitin column flow through. Chitin column fractions and fractions separated by anion-exchange chromatography were not tested for glucanase activity.

In contrast to the results with extracts of *S. simplex* seeds, neutralization of acid extract of *S. cubensis* seeds with 2 M NaOH did not denature the antifungal activity. Strong antifungal activity was present in the dialyzed neutralized acid extract (dialyzed in 10 mM Tris-HCl, pH 8.0 after neutralization). Antifungal activity bound to the column was eluted only with acetic acid. The sodium hydroxide eluate did not exhibit any antifungal activity. Strong antifungal activity was observed in the flow through of the acid chitin column.

Since the chitin-binding antifungal activity in neutralized acid extract only eluted at low pH and the chitin-binding activity in the neutral extract eluted with high pH, it is likely that the antifungal activities extracted using the two different extraction conditions are unique. Fractionation of acid extract (not neutralized but dialyzed in Tris-HCl, pH 8.0) by size-exclusion chromatography (Superose 12 column) did not separate the antifungal activity. Although the acid extract completely inhibited *A. flavus* growth, none of the fractions obtained after size-exclusion chromatography exhibited any antifungal activity against the same fungus. It appears that the antifungal components were bound to the column and were not eluted under these conditions.

Unless mentioned otherwise, *A. flavus* was used to assess the antifungal activity. One needs to keep in mind that these results may be different when tested against different fungi (see Chapter II for detailed discussion). Although purification of antifungal activities from seeds of *S. simplex* and *S. cubensis* were performed in parallel, antifungal activities purified from *S. cubensis* have not yet been characterized as well as purified antifungal proteins from *S. simplex*. For future studies, agglutination and glucanase assays need to be performed on the chitin column and anion-

exchange column fractions. If glucanases and/or agglutinins are detected, further purification of both can be based on the results obtained here. Testing against other fungi will also be worthwhile. Once this work is completed, efforts to clone and express the genes for these active components can be initiated.

CONCLUSIONS

Both neutral and acid extracts of seeds of *S. cubensis* contained potent inhibitors of fungal growth. Based on available evidences, at least some of these inhibitors are proteins. Some of the inhibitory activities bound to chitin and purified using a combination of chitin-affinity chromatography and anion-exchange chromatography. Antifungal activities eluted from the anion-exchange column fall into three groups. The first group strongly inhibited fungal growth, did not bind tightly to the RSQ column and contained a major protein band of about 41 kDa. This band was correlated with antifungal activity. The second group contained a few proteins with molecular weights in the range of 31-33 kDa. These proteins were chitinases and may account for the moderate inhibition of fungal growth observed in these fractions. The last group was tightly bound to the RSQ column and strongly inhibited fungal growth. A variety of protein bands were detected in the latter group under the conditions used herein.

Although the antifungal activity of the fractions that did not bind to chitin was very good, these activities have not been purified to date. Additional purification and characterization will be worthwhile.

Chapter VIII : Purification and Characterization of Antifungal Proteins from *Pentaclethra macroloba*

INTRODUCTION

Pentaclethra macroloba is one of the legumes found in the Costa Rican rainforest. Despite the diversity of predators and pathogens and the intensity of attack in the humid lowland tropical forest, *P. macroloba* has survived, flourished and established itself as one of the dominant tree species in these forests. In order to ensure successful germination, these plants must produce seeds that are able to protect themselves from pests and pathogens. In 1983, Hartshorn reported that seeds of *Pentaclethra macroloba* contained factors toxic to insects and small rodents.

The presence of trypsin inhibitors and other toxic factors in *P. macroloba* seeds was described by Schubert and Ruzicka (unpublished) in 1989. Two different trypsin inhibitors were isolated from *Pentaclethra macroloba* seeds by Chen et al. (1999). Both of the trypsin inhibitors reduced insect growth and increased larval mortality. Based on field

observations (K. R. Schubert, personal communication), seeds of *P. macroloba* remain on the forest floor for weeks without visible signs of fungal infection.

In our initial screening, crude extract and crude dialyzed extract of *P. macroloba* seeds (PCN # 1S) showed strong inhibition against *Fusarium chlamydosporum* growth. The results of our initial screen were supported by earlier results of Schubert and Duvick (personal communication). Their investigations showed that extracts of *P. macroloba* possessed antifungal activity against several pathogenic fungi. For these reasons, efforts were initiated to purify antifungal activities from this plant.

We have purified several antifungal chitinases from *S. simplex* and *S. cubensis* by affinity chromatography followed by ion-exchange chromatography. We wanted to find out, whether chitinases accounted for antifungal activity in extracts of *P. macroloba* seeds. Using the same purification scheme developed for isolation of chitinases from *Swartzia* seeds, a combination of chitin-affinity and anion-exchange chromatography was used to isolate chitinases and other antifungal activities from *Pentaclethra macroloba* seeds. The results of this purification were compared to results obtained using size-exclusion chromatography and anion-exchange chromatography.

MATERIALS AND METHODS

Antifungal assay

The antifungal assay was performed as described in Chapter III (Initial Screen). Extracts of *P. macroloba* seeds were tested for antifungal activity against *F. chlamydosporum*, *F. moniliforme* and *Aspergillus flavus*.

Extraction of *Pentaclethra macroloba* seeds

Three different extraction procedures were used in this study. Seeds were extracted with either 10 mM Tris-HCl (pH 8.0), 10 mM sodium phosphate (pH 7.5) or 0.1 M HCl. Frozen plant tissue was homogenized with Tris-HCl or phosphate buffer and PVP as described in previous chapters. In the case of HCl extraction, the crude extract was neutralized with 2 M sodium hydroxide by adjusting the pH to 3.8. After neutralization, the extract was either dialyzed or loaded onto the chitin-affinity column.

Size-exclusion chromatography

Pharmacia Sephacryl S-200 was packed into a 100-cm x 1.6-cm column according to the manufacturer's instructions. After the packing process was completed, the column was equilibrated with 10 mM sodium phosphate, pH

7.5. *Pentaclethra macroloba* seeds were extracted with 10 mM sodium phosphate, pH 7.5, and the crude extract was concentrated by ammonium sulfate precipitation. Solid ammonium sulfate was added to the extract slowly to a final concentration of 70% saturation. The pellet obtained after ammonium sulfate precipitation was resuspended in a minimum volume of 10 mM sodium phosphate (pH 7.5) and 2 ml of the resuspended pellet was loaded onto the column. The column was developed at a flow rate of 2 ml per min. Fractions were tested for antifungal activity against *F. chlamydosporum* and *F. moniliforme.*

Ion-exchange chromatography

Pharmacia S Sepharose resin was used for cation-exchange chromatography. The S Sepharose column was equilibrated with 50 mM sodium acetate buffer (pH 5.6). *Pentaclethra macroloba* crude extract was dialyzed in the same buffer and 20 ml of dialyzed extract was loaded onto the column. Unbound proteins were removed by washing with the starting buffer and the bound proteins were eluted with a linear gradient from 0 to 0.5 M NaCl in 50 mM sodium acetate buffer, pH 5.6, at a flow rate of 2 ml per min. Fractions were dialyzed in 10 mM Tris-HCl, pH 8.0 and assayed for antifungal activity.

Anion-exchange chromatography was also performed using a column packed with Pharmacia Q Sepharose resin. The column was equilibrated with 50 mM Tris-HCl, pH 8.0. *Pentaclethra macroloba* seeds were extracted with 10 mM Tris-HCl, pH 8.0 and the crude extract was dialyzed in the same buffer. A 20-ml sample of the dialyzed extract was loaded onto the Q Sepharose column. The column was washed with starting buffer to remove unbound proteins and bound proteins were eluted with a linear gradient from 0 to 0.5 M NaCl in the starting buffer at a flow rate of 2 ml per min. Fractions were dialyzed against 10 mM Tris-HCl, pH 8.0, and assayed for antifungal activity.

Anion-exchange chromatography on a Pharmacia RSQ column was used to further purify the acetic acid and sodium hydroxide eluates obtained after chitin-affinity chromatography. Anion-exchange chromatography was performed according to the methods described in Chapter IV.

Chitin-affinity chromatography

Chitin-affinity chromatography was carried out with neutralized acid extract according to the procedures for acid chitin column chromatography described in Chapter IV.

Other Methods

SDS-PAGE: SDS-PAGE was performed according to Laemmli (Laemmli, 1970) using a 12.5% polyacrylamide gel.

Protein determination: Protein concentration was measured with a dye-binding assay (Bradford, 1976). BSA was used as a protein standard.

Chitinase in-gel assay: Chitinase activity was determined using the chitinase in-gel assay according to the protocol described in Chapter IV.

Hemagglutination: Rabbit red blood cells (Sigma # R-1629) were used to detect agglutinins in chitin column and RSQ fractions. The assay method was the same as described for hemagglutination in Chapter IV.

Amino acid sequence analysis: Amino acid sequence was determined at the OUHSC Molecular Biology Resource Center (details in Chapter VI). Prior to sequencing, the sample was not HPLC purified.

EVALUATION OF PRELIMINARY PURIFICATION TECHNIQUES

Preliminary Purification

Size-exclusion chromatography (S-200), ion-exchange chromatography and chitin-affinity chromatography were evaluated as preliminary purification methods. The information gained from these preliminary purification procedures was used to develop a final purification scheme.

Size-exclusion chromatography

Fractions collected from the S-200 column chromatography were assayed for antifungal activity against *F. moniliforme* and *F. chlamydosporum*. The most active fractions were eluted with a higher elution volume. Inhibition of *F. moniliforme* was moderate in fraction 28 and strong inhibition was observed in fractions 26 and 27 (Figure VIII-1). Moderate activity against *F. chlamydosporum* was observed in many fractions. Fractions 26, 28 and 29 exhibited strong inhibition and very strong inhibition was observed in fraction 27. An analysis of the protein components in each fraction by SDS PAGE revealed several different bands (gel not shown) in the active fractions. No single band correlated with antifungal activity. Consequently,

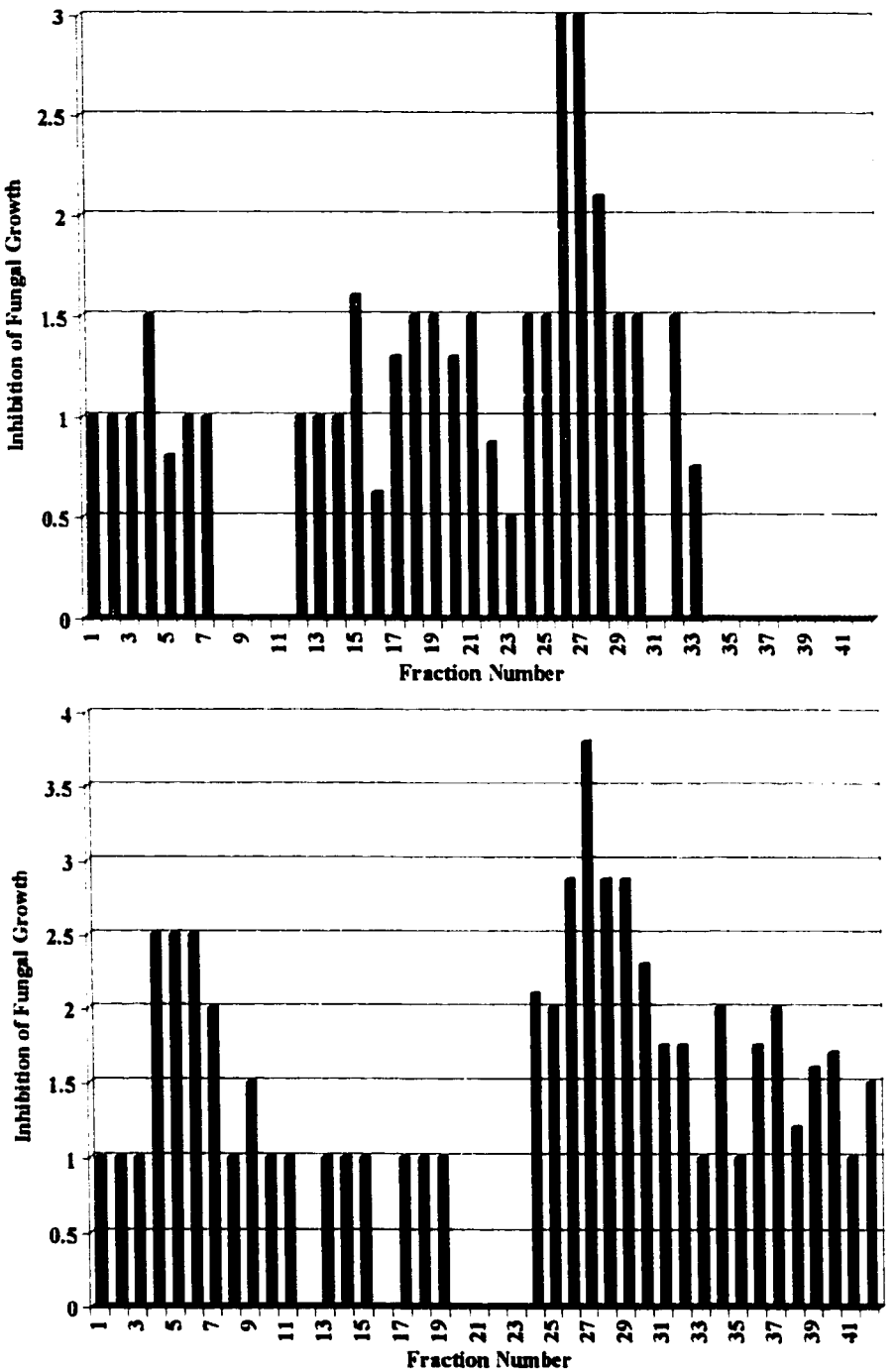


Figure VIII-1: Antifungal Activity of Neutral Extract Separated by Gel Filtration Chromatography. Seeds were extracted in 10 mM sodium phosphate, pH 7.5 and precipitated with ammonium sulfate. The pellet was resuspended in Na phosphate buffer and 2 ml of the resuspended pellet was fractionated on a S-200 column. Proteins were eluted with 10 mM sodium phosphate, pH 7.5, dialyzed in 10 mM Tris, pH 8.0, and tested for ability to inhibit growth of *F. moniliforme* (Top Panel) *and F. chlamydosporum* (Bottom Panel).

it appears that there is a range of antifungal activities in these extracts. These activities differ in their effects on the specific fungi tested.

Ion-exchange chromatography

Both anion and cation-exchange chromatography were evaluated as potential bulk purification methods. Seeds were extracted in 10 mM sodium phosphate, pH 7.5, or 10 mM Tris-HCl, pH 8.0, and dialyzed against the same buffer for cation and anion-exchange chromatography, respectively. Fractions from each column were dialyzed in 10 mM Tris-HCl, pH 8.0, and tested for antifungal activity against *F. moniliforme* and *F. chlamydosporum*. Antifungal activity did not elute, as a single peak but was spread out into many fractions after cation-exchange chromatography on the S-Sepharose column. In assays with *F. chlamydosporum*, moderate activity was observed in 22 fractions out of 28 fractions (data not shown). In the case of *F. moniliforme*, 6 fractions exhibited moderate inhibition and the rest of the fractions showed only slight activity (data not shown).

A similar pattern was observed after anion-exchange chromatography. All 28 fractions from the Q Sepharose column showed moderate inhibition

against *F. chlamydosporum* but did not inhibit the growth of *F. moniliforme.* These results suggest that more than one active component may be present in the extract and, therefore, ion-exchange-chromatography may be more effectively applied after some initial purification.

Chitin-affinity chromatography

Pentaclethra macroloba seeds (18 g) were extracted by homogenization in 5 volumes (90 ml) of 0.1 M HCl. Acid extract was neutralized with 2 N NaOH and centrifuged to remove denatured protein. After centrifugation, the clarified neutralized extract (10 ml) was loaded onto the chitin column equilibrated with 20 mM sodium acetate, pH 5.6. The flow through was reapplied to the column 3 times and the final flow through was retained. The column was washed as described previously and the bound proteins were eluted with 0.1 M acetic acid. The acetic acid eluate was pooled and the protein in the pooled fractions was precipitated with ammonium sulfate. The pellet obtained after ammonium sulfate precipitation (the eluate) was resuspended in 5 ml of 10 mM Tris-HCl, pH 8.0. Fractions eluted with 0.1 M sodium hydroxide were also pooled and precipitated with ammonium sulfate. The pellet was resuspended in 6 ml of 10 mM Tris-HCl, pH 8.0.

The neutralized HCl extract, flow through and all of the fractions from the chitin column were dialyzed against 10 mM Tris-HCl (pH 8.0) and assayed for antifungal activity using *A. flavus* and *F. moniliforme.* Strong antifungal activity against *A. flavus* was observed in the neutralized dialyzed HCl extract and also in the resuspended sodium hydroxide eluate (Figure VIII-2). Slight inhibition was present in the flow through. In the case of *F. moniliforme,* strong inhibition was observed in the neutralized acid extract, flow through and 0.1 M salt wash (data not shown).

Analysis of samples by SDS-PAGE revealed several protein bands in the acid extract, flow through, acetic acid eluate and sodium hydroxide eluate (Figure VIII-3). Two bands with estimated molecular weights between 31-32 kDa were very prominent in all these fractions. Results of the in-gel chitinase assay revealed several chitinase bands in the crude extract, flow through, acetic acid eluate and sodium hydroxide eluate (Figure VII-4).

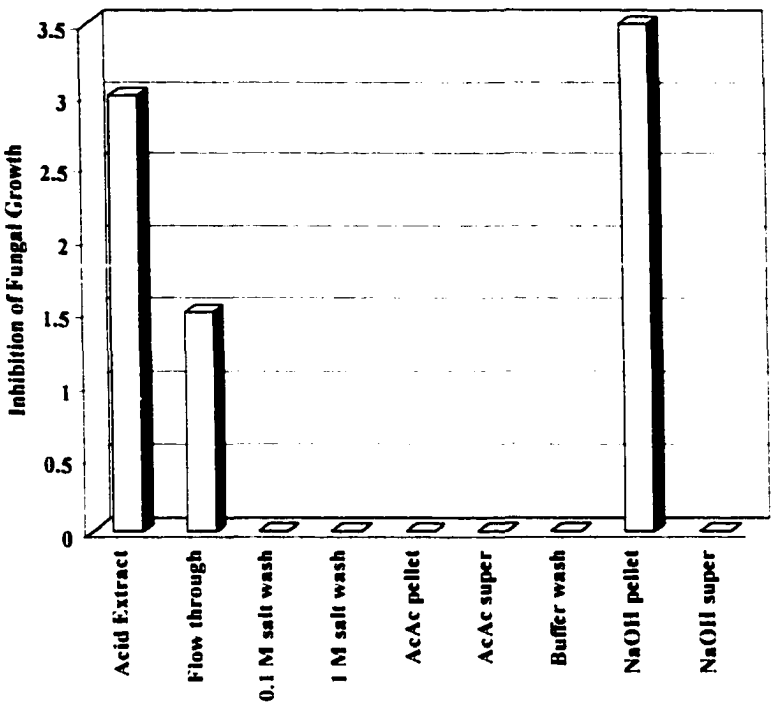


Figure VIII-2: Antifungal Activity of *P. macroloba* Acid Chitin Column Fractions. Ten ml of neutralized acid extract was fractionated on a chitin-affinity column. Fractions from chitin column were dialyzed against 10 mM Tris-HCl, pH, 8.0, and assayed for antifungal activity against *A. flavus*.

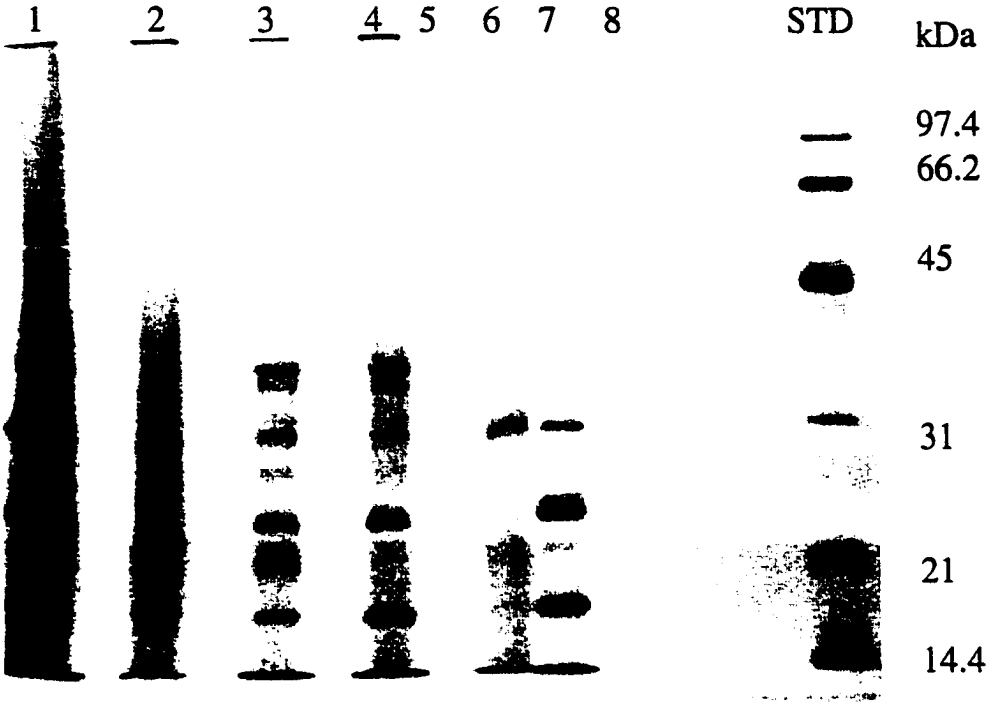


Figure VIII-3 : SDS-PAGE Gel of *P. macroloba* Acid Chitin Column Fractions. One hundred µl of each fraction was dried *in vacuo*, resuspended in 10 µl of SDS sample buffer and boiled for 5 min before loading onto a 12.5% SDS gel. Lane 1, flow through; lane 2, salt wash (0.1 M); lane 3, salt wash (1 M); lane 4, acetic acid eluate; lane 5, acetic acid supernatant; lane 6, salt wash (1 M); lane 7, sodium hydroxide eluate; lane 8, sodium hydroxide supernatant. The gel was silver stained to reveal protein bands. STD = molecular weight markers.

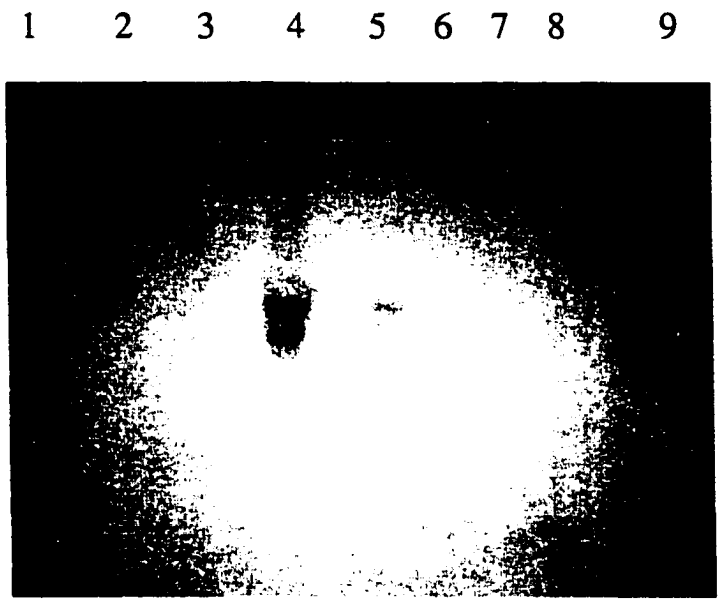


Figure VIII-4: Detection of Isoforms of *P. macroloba* Chitinases. Neutralized acid extract from *P macroloba* seeds was fractionated on a chitin column. A ten µl sample from each fraction was separated on a 12.5% native gel. Chitinase activity was detected using an in-gel assay. Lane 1, sodium hydroxide eluate; lane 2, sodium hydroxide supernatant; lane 3, 2nd Tris wash; lane 4, acetic acid eluate; lane 5, acetic acid supernatant; lane 6, salt wash (0.1 M); lane 7, salt wash (1 M); lane 8, flow through; lane 9, neutralized/dialyzed acid extract.

PURIFICATION OF CHITINASE AND OTHER ANTIFUNGAL ACTIVITIES

Purification of antifungal activity from the acetic acid eluate

The acid chitin column acetic acid eluate did not exhibit antifungal activity against *A. flavus*. Previously, we have shown that antifungal activity may be masked in more crude preparations. To test this possibility, the acetic acid eluate was fractionated by anion-exchange chromatography and the fractions obtained from the RSQ column were tested for antifungal activity.

Proteins in the acetic acid eluate obtained after chitin-affinity chromatography were concentrated by ammonium sulfate precipitation. The ammonium sulfate pellet was resuspended in a minimum volume of 10 mM Tris-HCl, pH 8.0, and dialyzed against the same buffer prior to anion-exchange chromatography. Four ml of the acetic acid eluate was injected onto a Pharmacia RSQ column equilibrated with 10 mM Tris-HCl, pH 8.0. The column was washed in the equilibrating buffer to remove unbound proteins and bound proteins were eluted with a linear gradient from 0 to 1 M NaCl in starting buffer.

The sample was separated into a number of discrete peaks (Figure VIII-5). Based on the absorbance profile, fractions were pooled, dialyzed and assayed for antifungal activity against *A. flavus*. Fractions 4 and 14 completely inhibited *A. flavus* conidial germination, while moderate inhibition was observed in fractions 11 and 13 (Figure VIII-6). SDS gel electrophoresis revealed a single (30-32 kDa) protein band in fraction 9 (Figure VIII-7). Two distinctly separated bands with molecular weights between 30 to 32 kDa were visible in fraction 10. The same size bands were present in fraction 11 but the upper band was much less abundant. Although antifungal activity was observed in fraction 14, protein bands were not visible in this fraction.

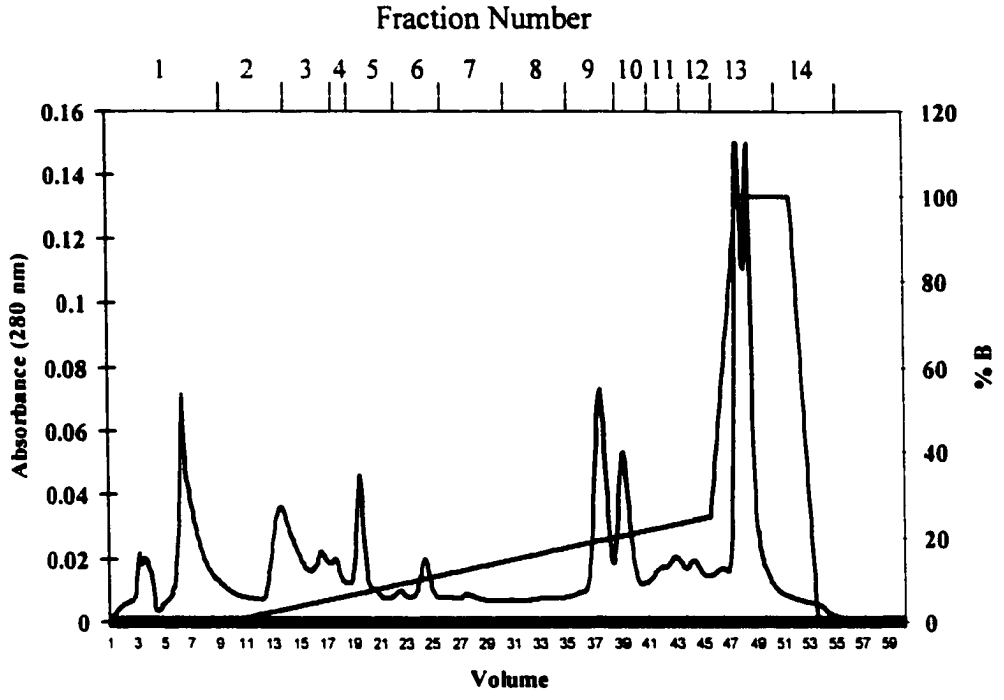


Figure VIII-5 : Absorbance Profile of Anion Exchange Column Chromatography of the Acetic Acid Eluate from the Acid Chitin Column. Four ml of acetic acid eluate was fractionated on a Pharmacia RSQ column. Bound proteins were eluted with a linear gradient of NaCl in 10 mM Tris-HCl, pH 8.0. Fractions were collected manually (as noted on the top of the figure).

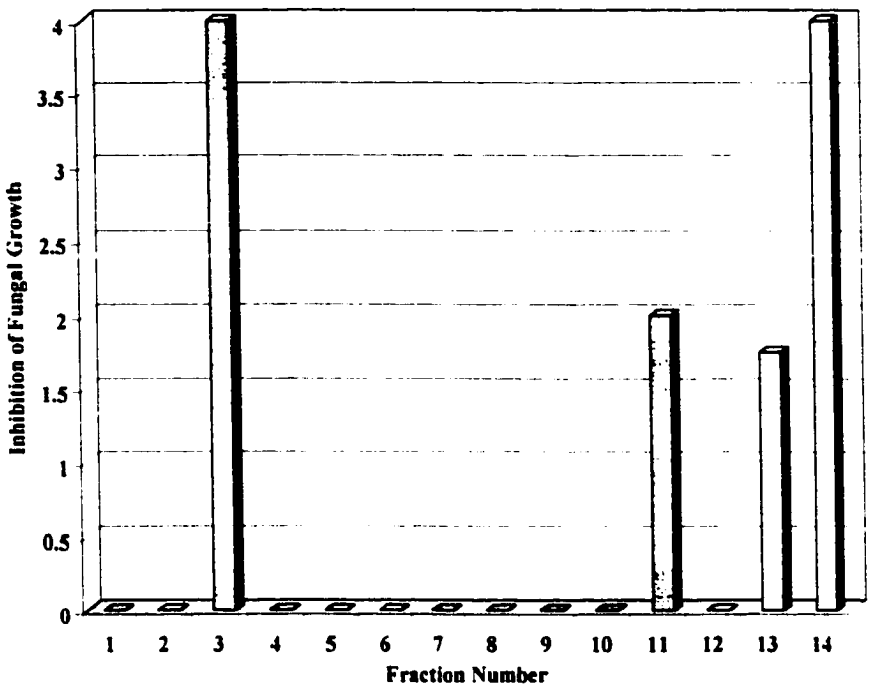


Figure VIII-6: Antifungal Activity of Fractions Obtained after Anion Exchange Chromatography of the Acetic Acid Eluate from the Acid Chitin Column. All fractions were dialyzed against 10 mM Tris-HCl, pH 8.0, and assayed for antifungal activity against *A. flavus* using the standard fungal bioassay.

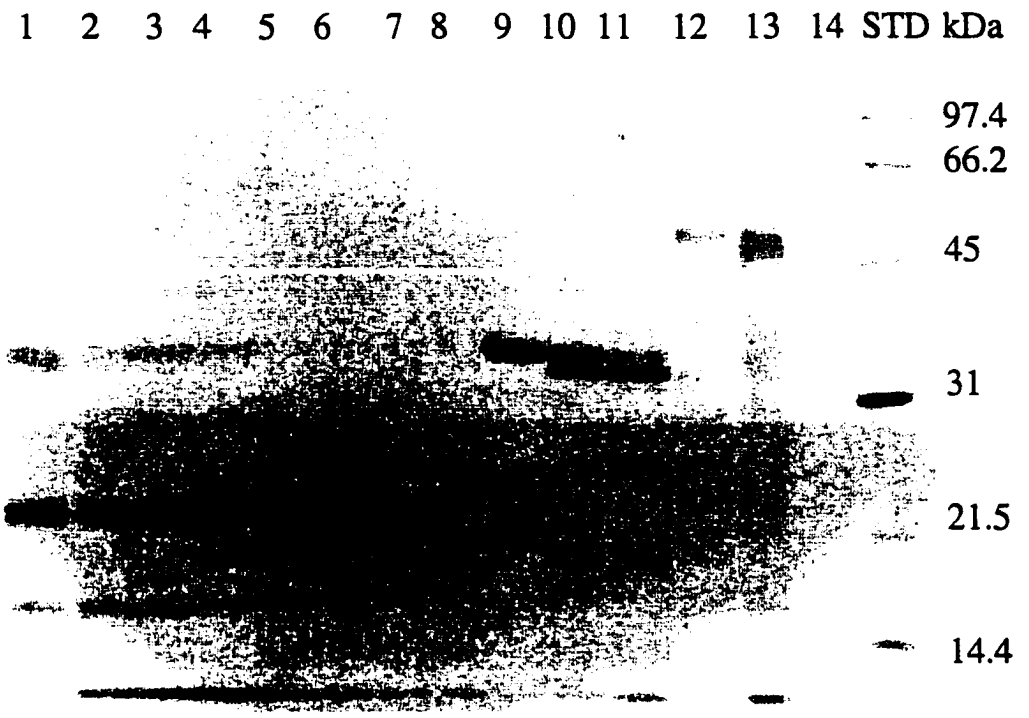


Figure VIII-7 : SDS-PAGE Analysis of Anion Exchange Column Fractions from the Acid Chitin Column Acetic Acid Eluate. Acid extract was fractionated on a chitin column. The acetic acid eluate from the chitin column was applied to an anion exchange (RSQ) column. A 200-µl sample from each fraction was dried *in vacuo*, resuspended in 10 µl of SDS sample buffer and boiled for 5 min before loading onto the 12.5% SDS gel. Lane 1 to 14, RSQ fractions (Figure VIII-5) 1 to 14. Gel was silver stained to reveal protein bands. STD = molecular weight standards.

Purification of antifungal activity from the sodium hydroxide eluate

Neutralized acid extract of *P. macroloba* seeds was applied to a chitin column and proteins were eluted as described previously. The resuspended pellet from the sodium hydroxide eluate was further fractionated by anion-exchange chromatography. The resuspended pellet was dialyzed against 10 mM Tris-HCl, pH 8.0, and 4 ml of this solution was loaded onto the column. The column was eluted using the same protocol as described for the acetic acid eluate. Fractions were pooled based on the A 280 profile (Figure VIII-8) dialyzed and assayed for antifungal activity against *A. flavus*. Fractions 3 and 4 completely inhibited conidial germination of *A. flavus* while fraction 12 caused moderate inhibition of hyphal growth (Figure VIII-9). The SDS gel profile was very similar to the SDS gel profile for the corresponding fractions after anion-exchange chromatography of the acetic acid eluate. A single protein band (30-32 kDa) was visible in Fractions 9 and 11 (Figure VIII-10). Fraction 10 contained two distinct protein bands with molecular weights between 31 and 32 kDa. Fractions 3 and 4 that exhibited antifungal activity contained a 22-kDa band. This band (marked with an asterisk in the figure) correlated with antifungal activity. A trace of the same band was also observed in fraction 5. Antifungal activity against *A. flavus* was not detected in the chitinase-containing fractions.

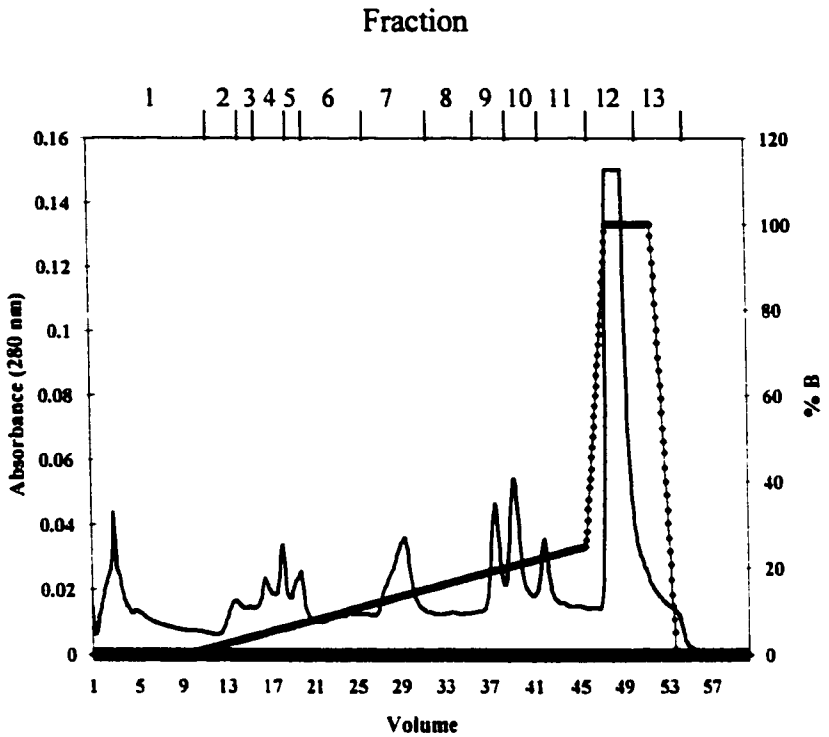


Figure VIII-8: Absorbance Profile of Anion Exchange Column Chromatography of Sodium Hydroxide Eluate from the Acid Chitin Column. Four ml of sodium hydroxide eluate from the acid chitin column was fractionated on a RSQ column equilibrated with 10 mM Tris-HCl, pH 8.0. Bound proteins were eluted with a linear gradient of NaCl in the same buffer. Fractions were collected manually based on the absorbance profile. The fractions are noted on the top of the figure.

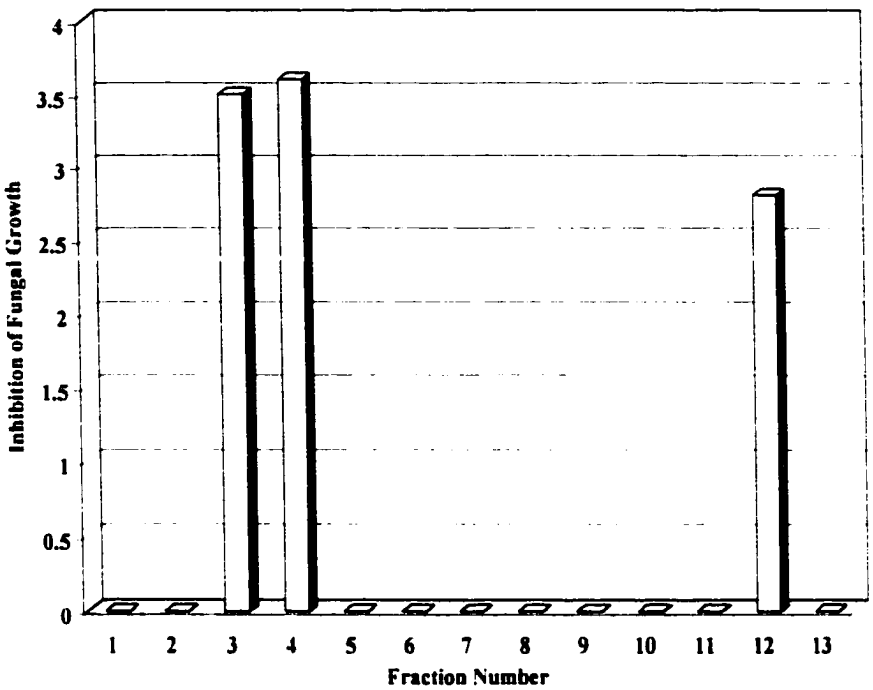


Figure VIII-9: Antifungal Activity of Fractions Obtained after Anion Exchange Chromatography of the Sodium Hydroxide Eluate from the Acid Chitin Column. Sodium hydroxide eluate from the acid chitin column was dialyzed in 10 mM Tris-HCl, pH 8.0, and applied to a RSQ column. All the fractions from anion exchange chromatography were dialyzed in 10 mM Tris-HCl, pH 8.0, and assayed for antifungal activity against *A. flavus*.

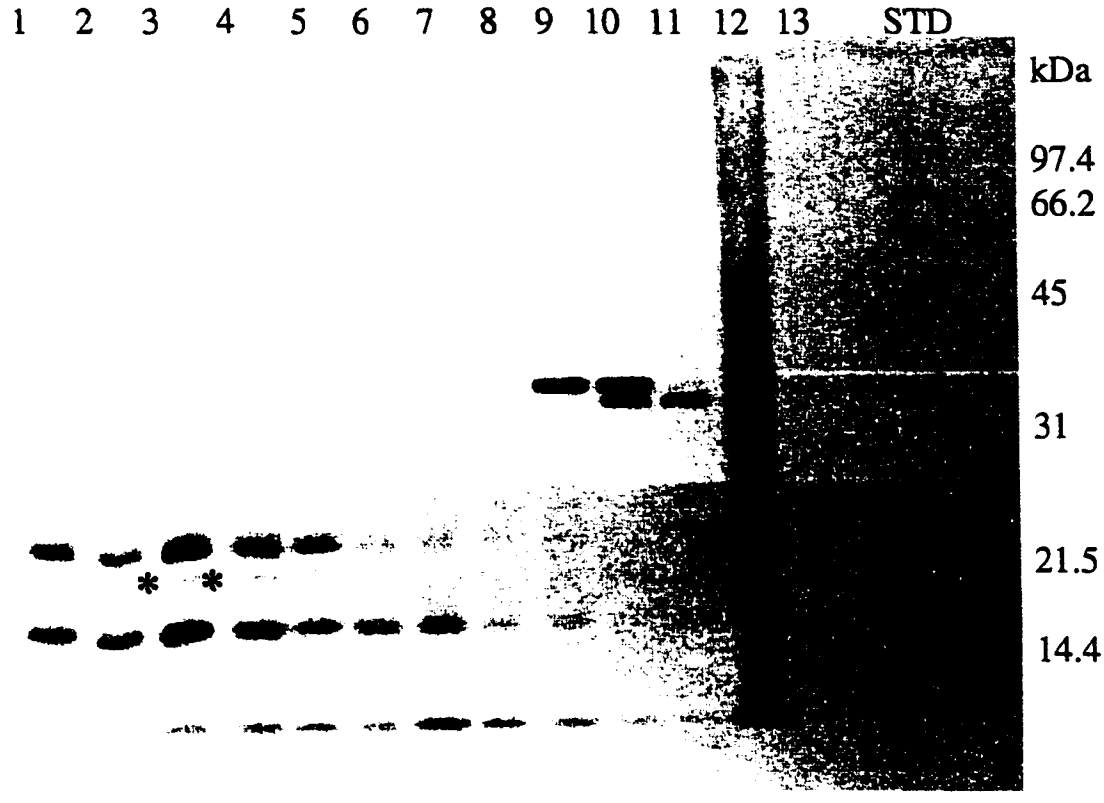


Figure VIII-10 : SDS-PAGE Analysis of Anion Exchange Column Fractions of the Sodium Hydroxide Eluate. Neutralized acid extract was fractionated on a chitin affinity column. The sodium hydroxide eluate from the chitin column was applied to an anion exchange (RSQ) column. A 200-µl of sample from each fraction was dried *in vacuo*, resuspended in 10 µl of sample buffer and boiled for 5 min before loading onto the gel. Lane 1 to 13, RSQ fractions (Figure VIII-8) 1 to 13. The gel was silver stained to reveal protein bands. Putative antifungal protein indicated with (*). STD = molecular weight standards.

CHARACTERIZATION

Detection of chitinase activity

Anion-exchange column fractions were tested for the presence of chitinase activity using the chitinase in-gel assay. Chitinase activity was detected in fractions 9, 10 and 11 obtained by anion-exchange chromatography of the acetic acid eluate from the chitin column (Figure VIII-11). From the activity gel profile, it was clear that at least four chitinase isoforms were present in Fractions 9 to 11. These activity bands corresponded to the protein bands revealed by SDS-PAGE. The purified chitinases from the acetic acid eluate were designated (in order of their increasing mobility on the activity gel) as A1 (fraction 9), A2 (fraction 10), A3 (fraction 10), A4 (fraction 11).

Chitinase activity was detected in the same fractions obtained after anion-exchange (RSQ) chromatography of the sodium hydroxide eluate (Figure VIII-12). Again, the in-gel assay revealed 4 different chitinase isoforms in these fractions which corresponded with the protein bands in fraction 9, 10 and 11. These chitinases were designated as chitinase B1 (fraction 9), B2 (fraction 10), B3 (fraction 10) and B4 (fraction 11) according to the order of increasing mobility on the chitinase activity gel.

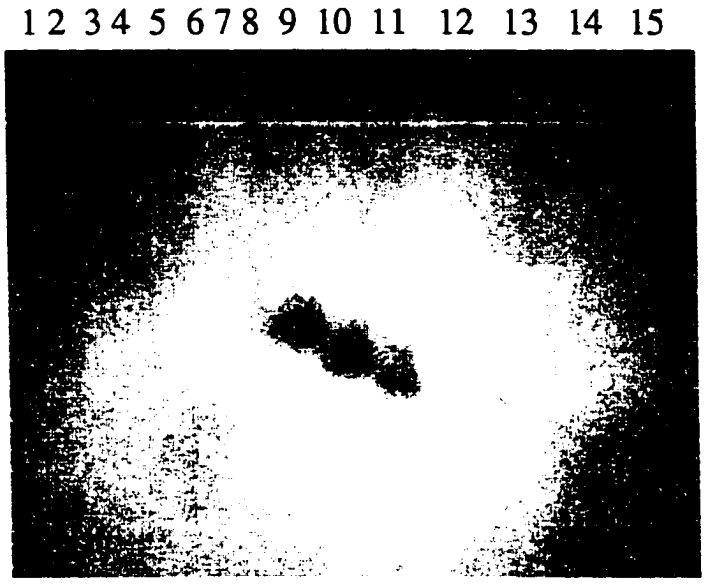


Figure VIII-11 : Detection of Isoforms of Chitinases after Anion Exchange Chromatography of the *P. macroloba* Acetic Acid Eluate. Acetic acid eluate from the acid chitin column was separated by anion exchange (RSQ) chromatography. A sample from each fraction was concentrated and 10 µl of the concentrated sample was separated on a 12.5% native gel. Chitinase activity was detected using the in-gel assay. Lane 1 to 14, fractions 1 to 14. Lane 15, positive control (Sigma chitinase).

RSQ Fraction Number

1 2 3 4 5 6 7 8 9 10 11 12 13

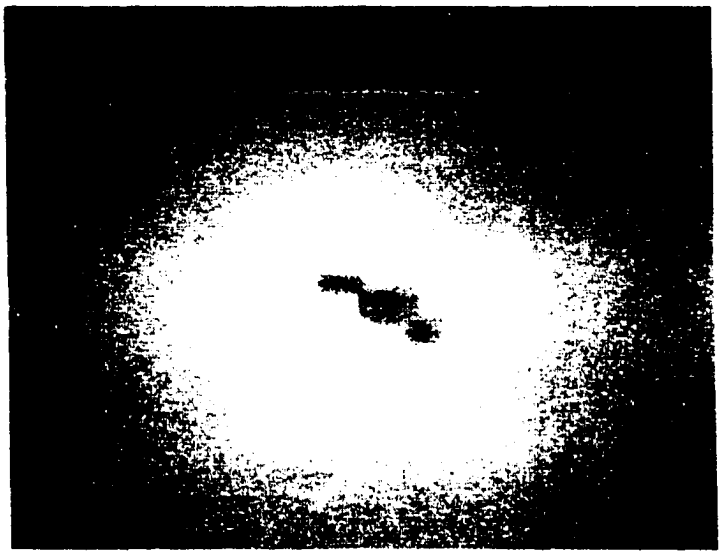


Figure VIII-12: Detection of Isoforms of Chitinases after Anion Exchange Chromatography of the *P. macroloba* Sodium Hydroxide Eluate. Sodium hydroxide eluate from the acid chitin column was separated by anion exchange (RSQ) chromatography. A sample from each fraction was concentrated and 10 µl of the concentrated sample was separated on a 12.5% native gel. Chitinase activity was detected using the in-gel assay. Lane 1 to 13, RSQ fractions 1 to 13.

Hemagglutination

The *P. macroloba* samples were tested for hemagglutination. The neutralized/dialyzed acid extract and flow through, 0.1 M salt wash, acetic acid eluate and sodium hydroxide eluate from acid chitin column agglutinated rabbit red blood cells (Figure VIII-13). The RSQ fractions from both the acetic acid eluate and sodium hydroxide eluate were also assayed for hemagglutination activity. Agglutination was not detected in any of the RSQ fractions from the acetic acid eluate even though, agglutination was observed in the starting material. Apparently, the agglutinin was bound tightly to the column and was not eluted from the column under the conditions used. Agglutination was observed in fraction 7, obtained after fractionation of the sodium hydroxide eluate on the RSQ column (Figure VIII-14). Agglutination in this case did not correspond with the antifungal activity in assays with *A. flavus*.

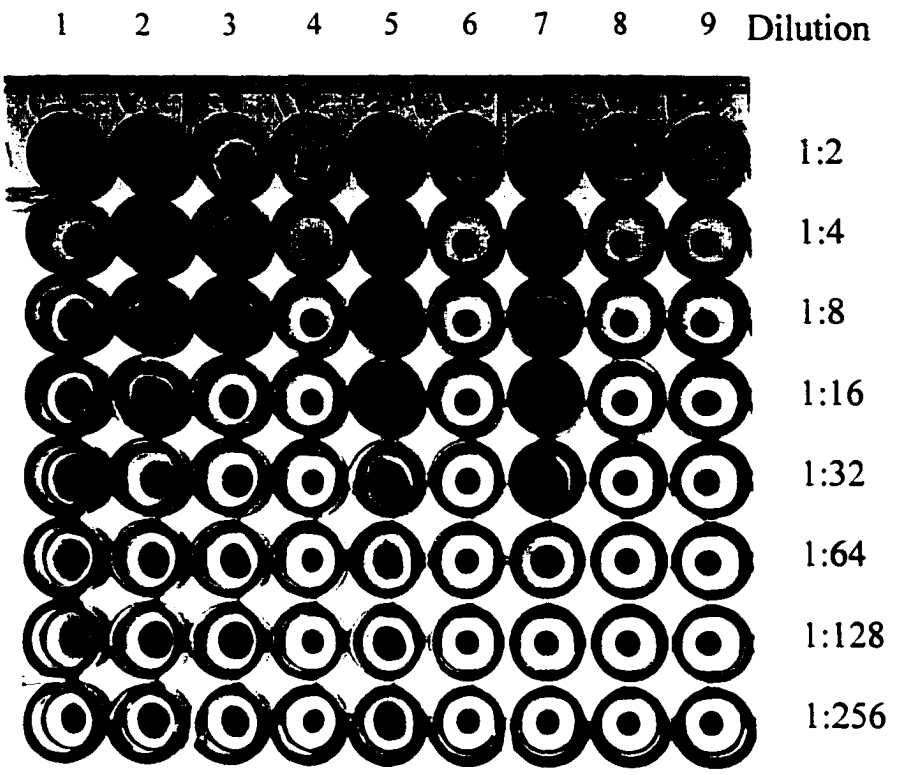


Figure VIII-13: Agglutination of Rabbit Red Blood Cells by Fractions after Chitin Affinity Chromatography of the *P. macroloba* Chitin Affinity Column. A sample (100-µl) from each fraction from the chitin affinity column was tested for agglutinating activity. Column 1, neutralized/dialyzed acid extract; column 2, flow through; column 3, salt wash (0.1 M); column 4, salt wash (1 M); column 5, sodium hydroxide eluate; column 6, buffer wash; column 7, acetic acid eluate; column 8, sodium hydroxide supernatant; column 9, acetic acid supernatant.

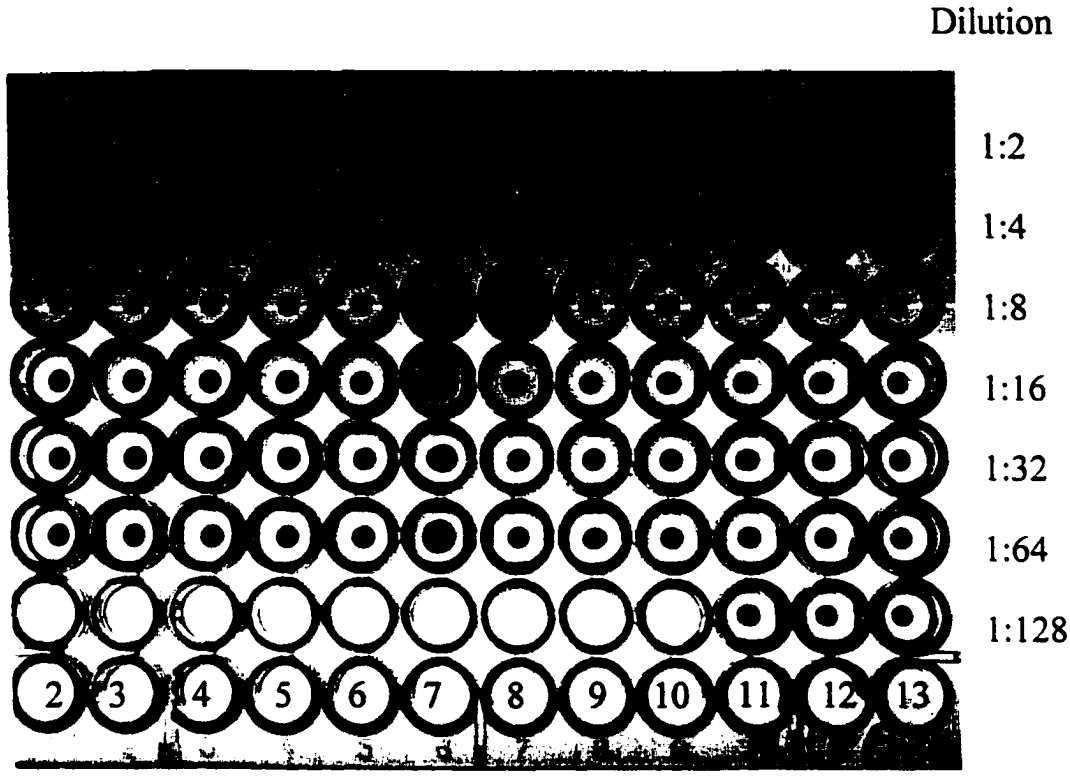


Figure VIII-14: Agglutination of Rabbit Red Blood Cells by Fractions after Anion Exchange Chromatography of the Sodium Hydroxide Eluate. Sodium hydroxide eluate from the acid chitin column was fractionated on a Pharmacia RSQ column. Fractions were dialyzed (10 mM Tris-HCl, pH 8.0) and a sample from each fraction was concentrated (2x). One hundred µl of the concentrated sample from each fraction was tested for hemagglutinating activity. Wells 2-13, RSQ column fractions 2-13.

Amino acid sequence analysis

Relatively pure chitinase was obtained after anion-exchange chromatography of the acetic acid and sodium hydroxide eluates from the acid chitin column. Fraction 11 (chitinase A4) was used directly for N-terminal sequencing. The N-terminal sequence of purified chitinase A4 was analyzed using a ProCise automated sequencer. Chitinase was not HPLC purified before sequencing. The N-terminal sequence for *P. macroloba* Chi A4 is:

Chi A4: KQIVTYWGQDVNQGKLDVAA

The N-terminal sequence obtained here was compared to the N-terminal sequences of other plant chitinases found in the Swiss Gene Bank Database using molecular biology tools available on the Internet. Comparisons of the N-terminal sequence revealed that chitinase A4 is highly homologous to chitinase class III chitinase/lysozyme precursor from several plant species including cucumber, azuki bean, Virginia creeper and para-rubber (Table VIII-1).

Table VIII-1: N-terminal Sequence Homology of *P. macroloba* Chitinase A4 with Chitinases from Other Plant Species.

Plant Species	Access number* (A)	% Identity	% Similarity	References
Cucumis sativus	p17541	57	71	Metraux et al., 1989
Phaseolus angularis	p29024	57	71	Ishige et al., 1993
Vitis vinifera	p51614	57	71	Busam et al., 1996
Parthenocissus quinquefolia	p23473	57	71	Bernasconi et al., 1987
Nicotiana tabacum	p29061	54	63	Lawton et al., 1992
Arabidopsis thaliana	p19172	57	71	Samac et al., 1990
Nicotiana tabacum	29061	78	85	Lawton et al., 1992
Nicotiana tabacum	p29060	64	78	Lawton et al., 1992
Hevea brasiliensis	p23472	57	71	Jekel et al., 1991
Cicer arietinum	p36908	52	64	Vogelsang and Barz, 1993
Beta vulgaris	p36910	60	80	Nielsen et al., 1993

* Swiss-Pro protein accession number.

DISCUSSION

Eight apparently different isoforms of chitinase were purified from extracts of *Pentaclethra macroloba* seeds using a combination of chitin-affinity and anion-exchange chromatography. The estimated molecular weights of these chitinases were between 31 and 32 kDa. The N-terminal amino acid sequence of chitinase A4 was determined and compared to the N-terminal sequences of other plants. This comparison revealed high sequence homology between *P. macroloba* chi A4 and class III chitinase/lysozyme from several other plant species. With the exception of the chitinase-containing fraction 11 obtained by anion-exchange chromatography of the acetic acid eluate, these purified chitinases did not exhibit any antifungal activity against *A. flavus* in the liquid assay used for assessment of antifungal activity.

Both the acetic acid eluate and sodium hydroxide eluate from the chitin-affinity column contained chitinases, chitin-binding agglutinin and antifungal activity. The chitin-binding agglutinins were separated from the fractions with antifungal activity and from chitinase-containing fractions by ion-exchange chromatography. Based on these findings it appears that the

chitin-binding lectins were not responsible for the antifungal activity against *A. flavus*.

Although in our assay, most of the purified chitinase from *P. macroloba* seeds did not show any antifungal activity against *A. flavus*, it is possible that these chitinases along with β-glucanase, may be active against *Aspergillus* or other fungi. In many cases, the antifungal activity of chitinases is highly selective. This is true in the case of antifungal chitinases from maize, wheat, pea and tobacco (Mauch et al., 1988; Verburg and Huynh, 1991; Huynh et al., 1992; Sela Buurlage et al., 1993). Even though these chitinases were tested against chitin-containing fungi, chitinases from these sources showed marked difference in activity. Chitinases from tobacco, thornapple and wheat inhibit growth of *Trichoderma hamatum* but not *Botrytis cineria* (Broekaert et al., 1988). Pea chitinase exhibited antifungal activity against *Thielaviopsis basicola* but not against *Aspergillus niger* or *Fusarium solani f. sp. phaseoli* (Mauch et al., 1988).

The amount of chitin varies as a percentage of the total fungal cell wall among the chitin-containing fungi (Rokem et al., 1986). According to Verberg and Huynh (1991), the susceptibility of different fungi to the same chitinase may depend on the amount of chitin in the fungal cell wall.

Therefore, it is quite possible, that the chitinases purified from *P. macroloba* seeds may exhibit antifungal activity against other chitin-containing fungi.

The biochemical nature of the antifungal activities from *P. macroloba* seeds has not been determined yet. Plants may contain a variety of pathogenesis-related proteins other than chitinases or chitin-binding lectins. Using chitin-affinity chromatography, Poinstein et al. (1994) purified a PR-4 protein containing a chitin-binding domain. The protein in *Pentaclethra macroloba* seed extracts responsible for antifungal activity against *A. flavus* may be a PR-4 protein, since the antifungal activity was bound to the chitin-affinity column but was not a chitinase or chitin-binding lectin. A 22-kDa band was visible in the RSQ fractions that inhibited growth of *A. flavus* (Fractions 3 and 4). This band was the only band visible that corresponded with the antifungal activity. Once isolated, a comparison of the sequence of this band to other antifungal proteins may help to reveal the nature of the protein.

CONCLUSIONS

Extracts of *P. macroloba* seeds exhibited antifungal activity against *A. flavus*, *F. chlamydosporum* and *F. moniliforme*. Antifungal proteins were purified from these extracts using chitin-affinity chromatography in combination with anion-exchange chromatography. Active proteins included chitin-binding proteins and proteins that did not have affinity for chitin. The N-terminal sequence of purified chitinase A4 from *P. macroloba* exhibited high sequence homology to class III chitinase/lysozyme from several plant species. In contrast to results with *S. simplex* and *S. cubensis* chitinases from *P. macroloba* were not very effective against A. flavus with the exception of Pm chitinase A4.

Chapter IX : Partial Purification of Antifungal Activity from *Coccoloba* sp.

INTRODUCTION

Extract from the fruit of PCN 98 was one of the most active of the tropical plant extracts identified in the initial screen for antifungal activity. Fruits of PCN 98 were collected from the lowland tropical rainforest of Costa Rica by a local naturalist, Mr. Orlando Vargas. According to the collector's field notes, PCN 98 was tentatively identified as a species from the genus *Coccoloba*. The later is a member of the Polygonaceae, or buckwheat family.

Crude extracts prepared from ripe *Coccoloba* fruits completely inhibited germination of *Fusarium chlamydosporum* conidia. Activity was retained after dialysis (3,500 MWCO). Out of the 163 extracts tested for antifungal activity, there were only two extracts that showed complete inhibition of fungal growth after dialysis and the extract from the fruit of *Coccoloba sp.* was one of them. However, there was a limited supply of these fruits. Because of the excellent antifungal activity found in this

extract, the decision was made to purify and characterize the antifungal activity as much as possible with the limited supply of material on hand.

MATERIALS AND METHODS

Extraction of plant tissue

Fruits from (4.38 g) *Coccoloba sp.* were extracted in 25 ml of 10 mM Tris-HCl buffer (pH 8.0) containing 0.2 g of insoluble PVP per g of tissue. All of the fruits used for extraction were ripe and rich burgundy in color. The plant tissue was homogenized using a Brinkmann PT-3000 homogenizer. The homogenized tissue was filtered through two layers of cheese cloth and two layers of Miracloth. The filtrate was centrifuged at 15,000 rpm for 15 min in a Sorvall SS 34 rotor. The supernatant fluid was collected and the pellet containing the insoluble material was discarded. The clear supernatant fluid referred to as the crude extract was highly pigmented, retaining the rich burgundy color. An aliquot of the crude extract was dialyzed extensively against 10 mM Tris-HCl buffer (pH 8.0) using a Spectra Por 3 membrane with a 3,500 Da molecular weight cut off. The dialyzed extract was clarified by centrifugation at 15,000 rpm for 15 min and the supernatant fluid was

retained (crude dialyzed extract). The crude and crude dialyzed extracts were tested for antifungal activity.

Antifungal assay

The antifungal bioassay was performed according to the procedure described in Chapter III using five different fungi. They were *Fusarium chlamydosporum*, *Fusarium moniliforme*, *Aspergillus flavus*, *Sclerotinia minor* and *Sclerotium rolfsii*. Purification of the active components was based on the results of the bioassay with *F. chlamydosporum*, *F. monoliforme* or *A. flavus*. The general bioassay protocol was modified slightly for assays with *S. minor* and *S. rolfsii*. Sclerotia of these two fungi were grown on potato dextrose agar (PDA) plates to obtain mycelial growth. After the desired growth of the mycelia, sclerotia were removed from the PDA plates and a single sclerotium was placed in the microtiter well containing growth media. Four replicates were used for each sample.

Working cultures of *S. minor* and *S. rolfsii* were maintained on PDA plates at room temperature. For long-term storage, cultures of *S. minor* were maintained as mycelial colonies on PDA plates at 4°C. For long-term preservation of *S. rolfsi*, cultures were grown on PDA slants, covered with sterile mineral oil and stored at room temperature. Sclerotia produced by these two fungi were also used for long-term storage. Stocks of both fungi

were kindly provided by Dr. Hassan Molouk from the Department of Entomology and Plant Pathology at Oklahoma State University.

Preliminary estimation of molecular weight

To obtain a preliminary idea about the size of the active component present in *Coccoloba* fruit extract, a sample of the dialyzed crude extract (3,500 Da MWCO) was dialyzed exhaustively using either 6-8,000 Da or 12-14,000 Da molecular weight cut off membranes. After dialysis, precipitated material was removed by centrifugation at 15,000 rpm for 15 minutes. The supernatant fluid was retained and tested for antifungal activity.

Heat treatment

To determine if the antifungal activity was heat stable, samples of crude dialyzed extracts were heated in a water bath for five min at 60, 70, 80, 90 or 100°C. After heat treatment, the extracts were cooled quickly on ice. To remove precipitated protein, the heat-treated samples were centrifuged at 15,000 rpm for 15 min. The pellet was discarded and the soluble material present in the supernatant was assayed for antifungal activity.

Ion-exchange chromatography

Both anion-exchange and cation-exchange chromatography was used to fractionate the antifungal activity present in the fruits of *Coccoloba*.

Anion-exchange chromatography: One ml of dialyzed crude extract was loaded onto a column (2 ml bed volume) packed with Pharmacia Q Sepharose and equilibrated with 10 mM Tris-HCl buffer (pH 8.0). After the sample was applied, the column was washed with the same buffer to remove unbound proteins. The flow through (FT) was collected and pooled. The proteins bound to the column were eluted with a step gradient of (2.5 ml each) 0.1 M, 0.2 M, 0.3 M, 0.4 M, 0.5 M and 2 M sodium chloride in 10 mM Tris-HCl buffer (pH 8.0). Fractions (2.5 ml) were collected and dialyzed against 10 mM Tris-HCl (pH 8.0) and all fractions were assayed for antifungal activity. Anion-exchange chromatography was also performed at a lower pH. In this case, the column was equilibrated with 10 mM Bis-Tris-HCl (pH 6.0). Crude extracts were dialyzed against the same buffer before loading 1 ml onto the column. Unbound proteins were collected and saved for antifungal assay as stated above. Bound proteins were eluted with a step

gradient of (2.5 ml each) 0.1 M, 0.2 M, 0.3 M, 0.4 M, 0.5 M, and 2 M sodium chloride in 10 mM Bis-Tris-HCl (pH 6.0). All the eluted fractions were collected in a volume of 2.5 ml, dialyzed separately in 10 mM Tris-HCl (pH 8.0) and assayed for antifungal activity.

Cation-Exchange Chromatography: Pharmacia S Sepharose resin was used for cation-exchange chromatography. The S Sepharose column (2 ml bed volume) was equilibrated with 10 mM HEPES (pH 8.0). Crude extract was dialyzed in the same buffer and 1 ml of the dialyzed extract was loaded onto the column. The column was washed with starting buffer to remove unbound proteins. The bound proteins were eluted with a step gradient of (2.5 ml each) 0.1 M, 0.2 M, 0.3 M, 0.4 M, 0.5 M and 2 M sodium chloride in 10 mM HEPES (pH 8.0). The flow through and all the collected fractions (2.5 ml each) were dialyzed in 10 mM Tris-HCl (pH 8.0) and assayed for antifungal activity.

Preparative isoelectric focusing

Preparative isoelectric focusing was performed with the neutral extract. Five ml of the dialyzed neutral extract was added to 25 ml of 20% glycerol. To this solution, 12.5 ml of ampholyte, pH range 3-10 (BioRad), was added

bringing the total volume of the solution to 42.5 ml. The final volume of the solution was brought to 50 ml by adding 7.5 ml of deionized water. This entire solution was injected into the rotofor cell and isoelectric focusing was carried out using the same protocol described in Chapter IV.

Affinity chromatography

Chitin-affinity chromatography was used to purify the antifungal activity from extracts of *Coccoloba* fruit. Sigma practical grade crab shell chitin was used as the column matrix. The chitin column (bed volume 20 ml) was prewashed extensively with 0.1 M HCl, deionized water, 0.1 M NaOH and 0.1 M NaCl in deionized water. The prewashed column was then equilibrated with 0.1 M sodium chloride in 50 mM sodium acetate buffer (pH 3.8).

Fruits were extracted in acid, using a slight modification of the method described by Peumans *et al.* (1983). All the fruits used for acid extraction were ripe, soft in texture and rich burgundy in color. Ten g of fruits were homogenized in 5 volumes of 0.1 M HCl. The homogenized tissue was filtered through two layers of cheese-cloth and Miracloth. The filtrate was centrifuged at 20,000 x g for 10 minutes. The supernatant fluid was collected and the pH was adjusted to 3.8 with the

addition of 2 N sodium hydroxide. After adjusting the pH to 3.8, the supernatant fluid was stirred on ice for one h, kept on ice for another h without stirring, and then centrifuged at 20,000 x g for 15 min. The supernatant fluid was saved and filtered through Whatman 3 MM filter paper before loading onto the chitin column. An aliquot of the supernatant fluid (referred to as acid extract) was exhaustively dialyzed against 10 mM Tris-HCl (pH 8.0) and the latter was tested in the antifungal bioassay.

Forty milliliters of the filtered acid extract was loaded onto the prewashed column. The flow through was collected and reapplied to the column four times. The final flow through was collected and assayed for antifungal activity. Unbound proteins were removed by washing the chitin column with 150 ml of 0.1 M and 1 M sodium chloride in 50 mM sodium acetate (pH 3.8) followed by 150 ml of 1 M NaCl in the same buffer. Bound proteins were eluted first with 0.1 M acetic acid. Fractions were collected in 10 ml volumes and the absorbance of the eluted proteins was measured at 280 nm. Fractions with an absorbance of 0.05 or greater were pooled and concentrated by ammonium sulfate precipitation. After addition of ammonium sulfate to a final concentration of 95% saturation, the solution was kept on ice and stirred until all the salt dissolved. The solution

containing ammonium sulfate was kept on ice without stirring for half an hour then centrifuged at 15,000 rpm for 30 min.

The supernatant fluid (65 ml) was saved and the pellet was resuspended in 1 ml of 10 mM Tris-HCl, pH 8.0. After acid elution, the chitin column was washed with 30 ml of 1 M sodium chloride in 50 mM sodium acetate (pH 3.8). At this point, the column was eluted with high pH (0.1 M NaOH) to remove proteins still bound to chitin. The base eluted fractions were pooled, ammonium sulfate precipitated and centrifuged according to the protocol used for acid-eluted fractions. After centrifugation, the supernatant fluid (50 ml) was saved and the pellet was resuspended in 5.0 ml of 10 mM Tris-HCl (pH 8.0). The three salt washes, the acetic acid and sodium hydroxide-eluted ammonium sulfate-precipitated pellets, and supernatants after ammonium sulfate precipitation were exhaustively dialyzed in 10 mM Tris-HCl (pH 8.0) and assayed for antifungal activity.

Chromatography on P-10 resin

The ammonium sulfate pellet from the NaOH-eluted fractions obtained from the chitin column was resuspended in 10 mM Tris-HCl, pH 8.0. A total of 4 ml of this was loaded onto a BioRad P-10 column (1.5-cm x 30-cm). The

column was equilibrated with 0.02 M sodium phosphate buffer (pH 7.0) and proteins were eluted with the same buffer at a flow rate of 2 ml/min. Twenty-four fractions, including the void volume, were collected in 5 ml volumes. The absorbance of the eluted proteins was measured at 280 nm. Every four fractions were pooled resulting in seven 20-ml fractions. The column was then washed with 0.1 M NaOH to elute the pigmented part of the sample that was still bound to the column. The 0.1 M NaOH wash was collected in two fractions (20 ml/fraction). All of the fractions from the P-10 column were dialyzed against 10 mM Tris-HCl, pH 8.0. After dialysis, 2 ml of each fraction was concentrated by ultrafiltration using a 10,000 Da MWCO Centricon concentrator (Amicon). Both the retentate and filtrate were saved and assayed for antifungal activity.

Size-exclusion chromatography

The pigmented and non-pigmented components from the crude dialyzed extract were separated first on a P-10 column. Sixty-seven ml of the non-pigmented portion was concentrated by ammonium sulfate precipitation (95% saturation). The precipitated pellet was resuspended in 1 ml of 10 mM sodium phosphate buffer (pH 7.0) and 0.5 ml of this resuspended pellet was loaded onto a Pharmacia Sephacryl S-200 column. The column was packed

according to the protocol provided by Pharmacia and equilibrated with 10 mM sodium phosphate buffer (pH 7.0). After application of the sample, the column was eluted at a flow rate of 1 ml/min. Thirty-six 2-ml fractions were collected. All of the fractions from S-200 column were dialyzed in 10 mM Tris-HCl (pH 8.0) before testing for antifungal activity.

SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out under denaturing conditions according to Laemmli using 7.5%, 12.5%, and 15% acrylamide concentrations (Laemmli, 1970). Samples were denatured by heating at 100°C for five min in SDS sample buffer containing 2 mM β-mercaptoethanol. Unless otherwise mentioned, electrophoresis was carried out at constant voltage for 3-4 hours. Gels were stained with Coomassie Brilliant Blue (CBB) R-250. After staining, protein bands were revealed by destaining gels in 30% methanol: 10% acetic acid: 60% water (v/v/v) for 10-24 h. In some cases, gels were restained with silver nitrate to achieve greater sensitivity of protein detection (Bloom, 1987).

Isoelectric focusing gel electrophoresis

A BioRad mini IEF apparatus was used to perform IEF gel electrophoresis. To prepare the IEF gel solutions, one ml of acrylamide solution (20% acrylamide, 3% bisacrylamide [BioRad]), 1 ml of 25% glycerol (v/v) and 0.5 ml of ampholyte (Biolyte with a pH range of 3-10) were mixed with 2.5 ml of deionized water. The solution was degassed and then 50 µl of freshly prepared 10% (w/v) ammonium persulfate, 50 µl of 0.1% FMN and 5 µl TEMED were added to the solution.

The gel solution was poured into the chamber and allowed to polymerize for one h. Protein samples (4 µl) were loaded on to the thin polyacrylamide gel. The gel was pre-run at 100 and 200 volts for 15 min each. IEF was then performed at 400 volts for one h. After electrophoresis the gel was stained with CBB R-250 containing 0.5% $CaSO_4$, $5H_2O$ and 50 mg Crocein Scarlet 7B (Sigma # C 8822). After Staining the gel for 1 h, the gel was destained in destaining solution overnight and then stained with silver nitrate.

RESULTS

The crude extracts prepared from the fruits of *Coccoloba sp.* inhibited the growth of selected pathogenic and/or saprophytic fungi (Table IX-1). This extract completely inhibited conidial germination of *Sclerotinia minor*, *Sclerotium rolfsii*, *F. moniliforme* and *F. chlamydosporum* even after exhaustive dialysis. There was a very strong inhibition of *A. flavus* growth (rating 3.8) in assays containing crude or crude dialyzed extracts. Antifungal activity was retained even after exhaustive dialysis using 6-8,000 and 12-14,000 Da MWCO dialysis membranes (Table IX-2). The dialyzed extracts exhibited very strong to complete inhibition of growth of both *F. chlamydosporum* and *F. moniliforme.*

The dialyzed extracts were heated at 60, 70, 80, 90 and 100°C for five min. The antifungal activity present in *Coccoloba sp.* fruit extract was heat stable. Even after boiling for five min, the dialyzed extract completely inhibited growth of *F. moniliforme* and *F. chlamydosporum* (Table IX-3).

Table IX-1: Antifungal Activity of Extracts of *Coccoloba* with Different Fungal Pathogens.

FUNGAL PATHOGEN	Inhibition of Fungal Growth		
	Crude Extract	Dialyzed Extract	Buffer Control
Fusarium moniliforme	4.0	4.0	0
Fusarium chlamydosporum	4.0	4.0	0
Aspergillus flavus	3.8	3.8	0
Sclerotinia minor	4.0	4.0	0
Sclerotium rolfsii	4.0	4.0	0

Extracts were tested for activity against various pathogenic and/or saprophytic fungi. Fungal growth ratings for each fungus are presented.

Table IX-2: Preliminary Estimation of Molecular Weight of Antifungal Activity of *Coccoloba* sp.

FRACTION	INHIBITION OF FUNGAL GROWTH	
	F. moniliforme	*F. chlamydosporum*
Crude Neutral Extract	4.0	4.0
Dialyzed 3,500 MWCO	4.0	4.0
Dialyzed 6-8,000 MWCO	4.0	4.0
Dialyzed 12-14,000 MWCO	3.8	3.8
Buffer Control	0	0

The molecular size of the antifungal activity from *Coccoloba* sp. was estimated based on retention after exhaustive dialysis. Samples were dialyzed using membranes with different molecular weight cut off (MWCO) ranges. Dialyzed material retained within the membrane was tested for antifungal activity in the bioassay using *Fusarium moniliforme* and *F. chlamydosporum*.

Table IX-3: Effects of Heat Treatment on Antifungal Activity.

Fraction	INHIBITION OF FUNGAL GROWTH		
	F. moniliforme	*F. chlamydosporum*	*A. flavus*
Crude	4.0	4.0	4.0
Dialyzed	3.9	4.0	3.9
60 C	3.9	4.0	3.5
70 C	3.9	4.0	3.9
80 C	4.0	4.0	3.9
90 C	4.0	4.0	3.9
100 C	4.0	4.0	3.9
Buffer	0	0	0

Extracts were heated at the designated temperatures for 5 min and centrifuged to remove denatured protein. Fungal growth inhibition ratings for each treatment are presented for three different fungi.

To determine whether the activity was stable at low pH, fruits were extracted with 0.1 M HCl and the acid extract was dialyzed to remove the HCl. The activity was stable in 0.1 M HCl. acid. The dialyzed HCl extract in 10 mM Tris-HCl, pH 8.0, completely inhibited the growth of both *F. moniliforme* and *A. flavus* (Figure IX-1).

Many antifungal proteins bind to chitin and can be purified by chitin-affinity chromatography. To determine whether the active components in extracts of *Coccoloba* fruits also had chitin-binding activity, fruits were extracted with 0.1 M HCl. After extraction, the pH of the acid extract was adjusted to pH 3.8. This material was applied to a chitin-affinity column and chitin-binding components were eluted using 0.1 M acetic acid and 0.1 M sodium hydroxide. After elution, proteins in the acid eluate and sodium hydroxide eluate were precipitated with ammonium sulfate and resuspended in 10 mM Tris-HCl, pH 8.0. Although the acetic acid eluate did not inhibit fungal growth, the fraction obtained after elution with 0.1 M NaOH exhibited very strong inhibition against *F. moniliforme* and *A. flavus* (Figure IX-1).

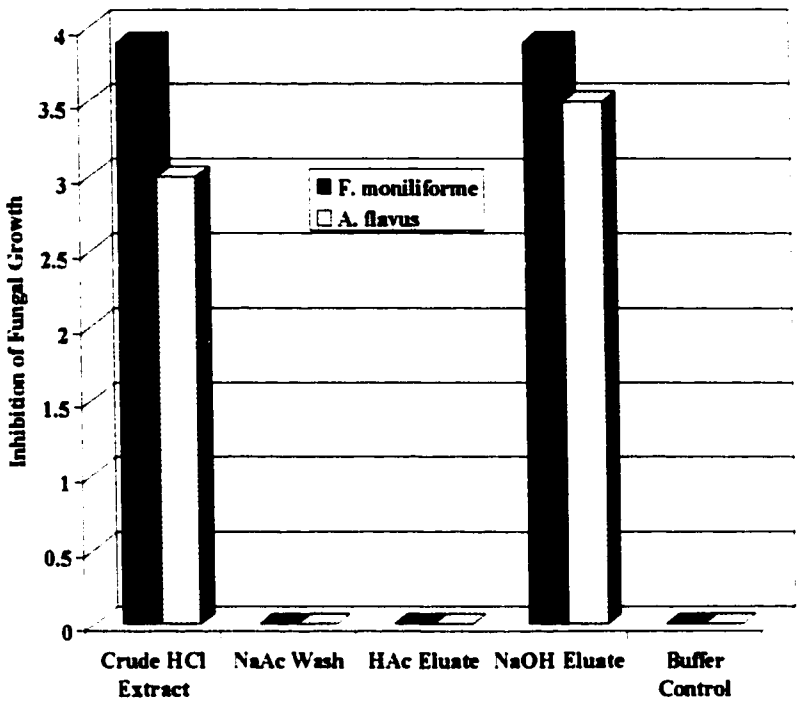


Figure IX-1: Antifungal Bioassay of Fractions from the Chitin-Affinity Column. Neutralized acid extract was fractionated by chitin-affinity chromatography. A sample from each fraction was tested for antifungal activity with *A. flavus* and *F. moniliforme*.

The active fractions (NaOH eluate) from the chitin-affinity column were highly pigmented. The color of the resuspended pellet of the NaOH-eluted fractions resembled the rich burgundy color of *Coccoloba* fruits.

The nature and purity of proteins in the active and inactive fractions were analyzed using denaturing gel electrophoresis (SDS-PAGE). Fractions from the chitin-column were subjected to SDS-PAGE using gels with three different concentrations of acrylamide. Unfortunately, the heavy pigmentation caused extensive streaking and interfered with the detection of individual protein bands on these gels. In an attempt to clarify the fractions and to remove interference caused by pigmented substances, the material was precipitated with acetone prior to SDS-PAGE (Remy and Ambard-Bretteville, 1987). There was no pellet visible after acetone precipitation and no protein bands were detected after performing SDS-PAGE with the acetone-precipitated sample.

The protein-banding pattern of individual fractions was analyzed by IEF slab gel electrophoresis (gel not shown). IEF gel electrophoresis revealed several protein bands in crude dialyzed neutral extract (extracted and dialyzed in 10 mM Tris-HCl, pH 8.0). Four bands were detected in the fraction that was eluted from the chitin-affinity column with sodium

hydroxide and precipitated with ammonium sulfate. In contrast, no protein bands were detected in the fraction eluted with acetic acid and precipitated with ammonium sulfate. This was consistent with the absence of a visible pellet, after ammonium sulfate precipitation of the acetic acid eluate.

Pigmentation of the extract also interfered with subsequent purification. When glycerol was added to the dialyzed crude extract prior to preparative isoelectric focusing, the pigmented material precipitated. This eventually interfered with the collection of the samples after IEF. The precipitated pigments clogged part of the collection tubes resulting in very uneven collection of samples. Normally, each fraction would contain 2 to 2.5 ml. In the present situation, some tubes had less than 0.5 ml while other tubes had more than 4 ml of sample. In addition, it was not possible to determine an accurate pH profile for the fractionation. Even so, the collected samples were clear and devoid of any pigments. To our dismay, when these samples were assayed, there was no inhibition of *F. chlamydosporum* or *A. flavus* growth in any of the fractions (data not shown).

Our initial conclusion from these experiments was that the activity was associated with the pigmented materials. To test this possibility, it was necessary to determine with certainty whether the activity was associated

with the pigmented or the non-pigmented portion. To separate the pigmented substances from the non-pigmented components, the active fraction after chitin-affinity chromatography was fractionated by size-exclusion chromatography on a Biogel P-10 column.

Using this technique, the pigmented portion was successfully separated from the non-pigmented portion. The non-pigmented fraction was eluted easily with 0.02 M sodium phosphate buffer (pH 7.0). The pigmented portion remained bound to the column even after the column was washed with 1 M NaCl in sodium phosphate buffer (pH 7.0), lithium hydroxide or acetic acid. Eventually, the very hydrophobic pigmented material was successfully eluted in two fractions with 0.1M NaOH. The 24 non-pigmented fractions from the P-10 column obtained after elution with sodium phosphate were pooled into six fractions and the pigmented fractions eluted with NaOH (PN1 and PN2) were kept separate. PN1 was rich burgundy brown in color indicating a very high concentration of pigments. PN2 had a lower concentration of pigment and was light pink. According to the antifungal assay (Figure IX-2), there was antifungal activity in all six of the pooled non-pigmented fractions. These fractions strongly inhibited growth of *F. moniliforme* and *A. flavus* mycelia. The pigmented fractions,

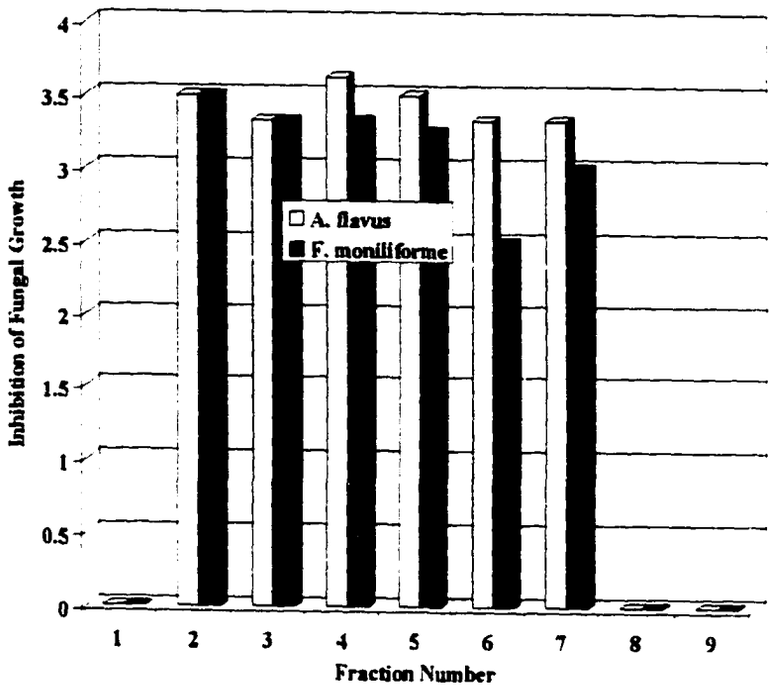


Figure IX-2: Separation of Antifungal Activity by Size Exclusion Chromatography on Biogel P-10. Sodium hydroxide eluate from the chitin affinity column was fractionated on a P-10 column. Non-pigmented fractions 1 to 7 were eluted with phosphate buffer. Pigmented fractions 8 (PN1) and 9 (PN2) were eluted with sodium hydroxide. Fractions were dialyzed and assayed for antifungal activity.

on the other hand, had no antifungal activity against either of the fungi mentioned in the section above. A 12.5% SDS-PAGE gel of the non-pigmented fractions revealed the presence of protein bands in all of the fractions (Figure IX-3).

Several other approaches to protein purification were evaluated during the initial phases of purification protocol development. These included ion-exchange chromatography on anion and cation-exchange columns and size-exclusion chromatography. Crude dialyzed extract of *Coccoloba* fruits was applied to a Pharmacia Q Sepharose column equilibrated with 0.1 M Tris-HCl buffer, pH 8.0. Bound proteins were eluted with a step gradient from 0 to 2.0 M NaCl in the same buffer. Fractions from the Q Sepharose column were dialyzed against 10 mM Tris-HCl, pH 8.0, to remove salt and tested for activity in the fungal bioassay using *F. moniliforme* and *A. flavus* (Figure IX-4). Activity was not detected in the flow through or in any of the column fractions, suggesting that the active component remained tightly bound to the column. Consistent with the results of anion-exchange chromatography

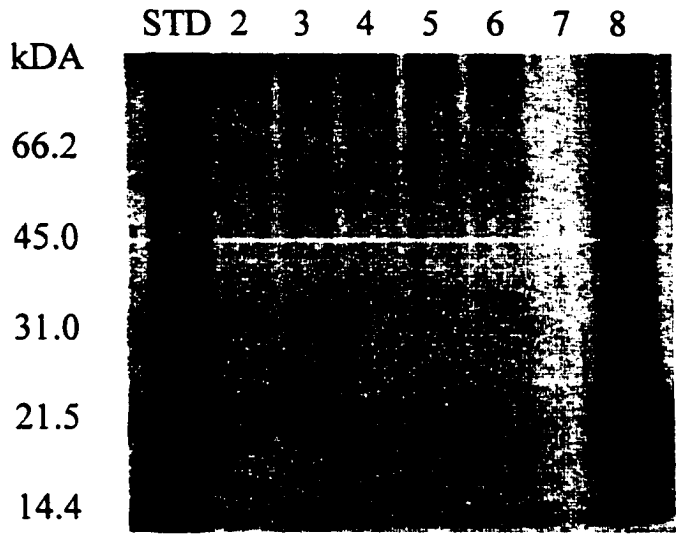


Figure IX-3: Analysis of Protein Banding Patterns of P-10 Fractions by SDS-PAGE. Lanes 1-7 were active fractions obtained by fractionation on a Biogel P-10 column .

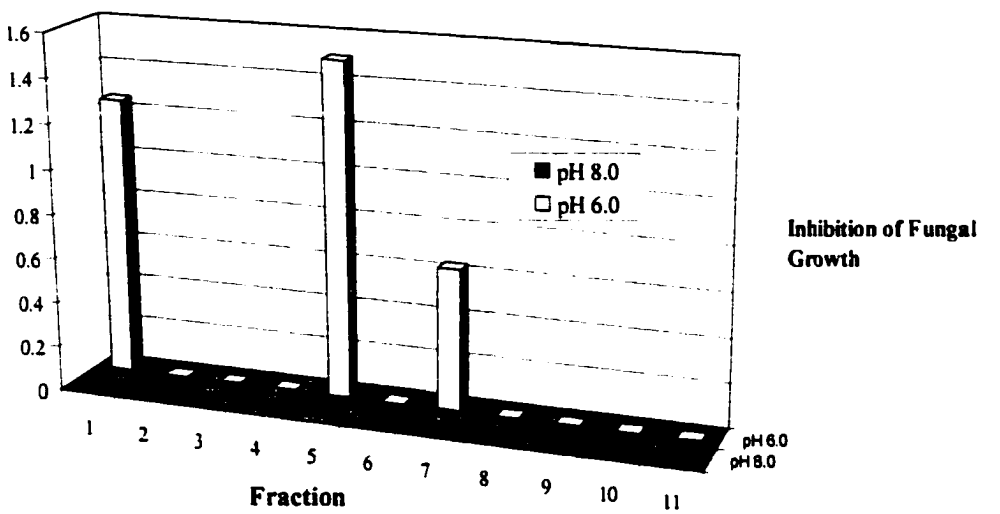


Figure IX-4: Fractionation of Antifungal Activity by Anion-Exchange Chromatography on Q Sepharose. Dialyzed neutral extract of Coccoloba fruits were fractionated on a Q Sepharose column at pH 6.0 and pH 8.0. Bound proteins were eluted with a step gradient of NaCl. Collected fractions were dialyzed in 10 mM Tris-HCl, pH 8.0, and assayed for antifungal activity using *A. flavus*.

at pH 8.0, the antifungal activity was not retained on a Pharmacia S Sepharose strong cation-exchange column equilibrated with 10 mM HEPES at pH 8.0. The unbound fraction (flow through) exhibited very strong inhibition against both *A. flavus* and *F. moniliforme* (Figure IX-5). These results support the conclusion that the active material is acidic and is highly negatively charged at pH 8.0. Based on the assumption that the protein was highly negatively charged (i. e. the pI was acidic), crude dialyzed extract was applied to a Q Sepharose column equilibrated with 20 mM Bis-Tris-HCl at pH 6. This should reduce the strength of binding. In this case, some activity came through in the flow through and wash, while another peak of activity was eluted with moderate concentrations of NaCl (Figure IX-4).

Crude dialyzed extract was concentrated by ultrafiltration and subjected to size-exclusion chromatography on Pharmacia Sephacryl S-200. Individual fractions were tested for antifungal activity using *F. moniliforme* and *A. flavus*. The activity was observed in almost all of the fractions (data not shown). Fraction 1 to 21 and 35 exhibited very strong inhibition of *F. moniliforme* growth while all 36 fractions exhibited strong inhibition of *A. flavus* growth.

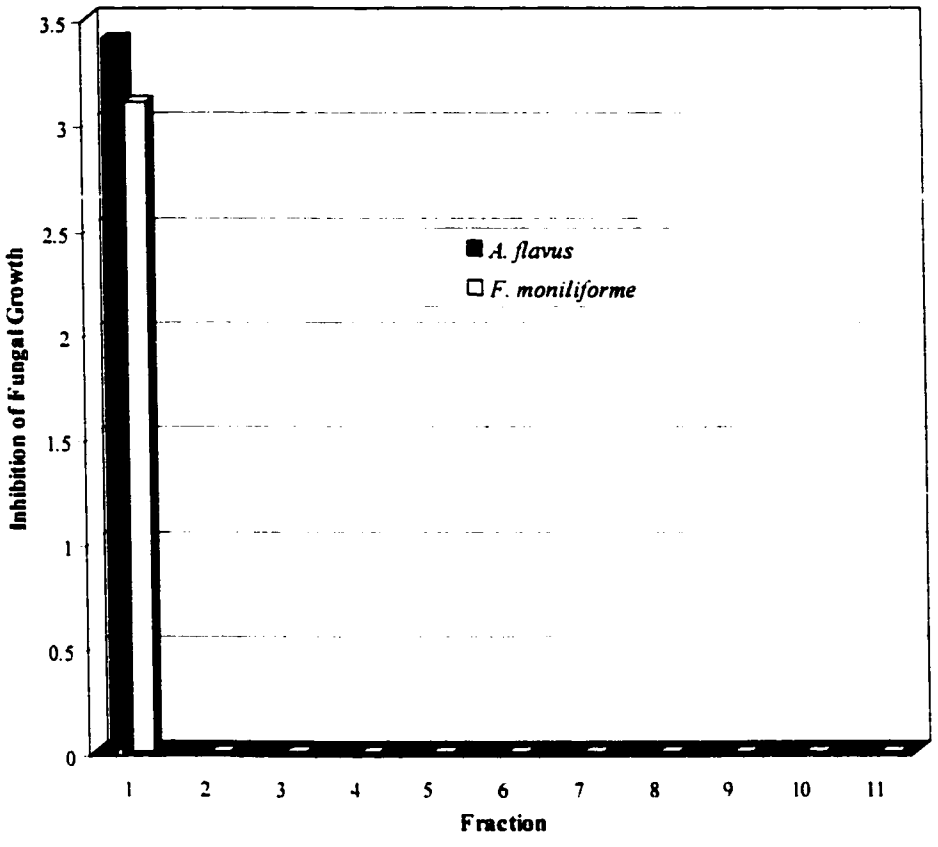


Figure IX-5: Fractionation of Antifungal Activity by Cation Exchange Chromatography on S Sepharose. Bound proteins were eluted with a step gradient of NaCl. Fractions were dialyzed in 10 mM Tris pH, 8.0, and assayed for antifungal activity against *A. flavus* and *F. moniliforme*.

DISCUSSION

PCN 98 was collected by Mr. Orlando Vargas. Unfortunately, Mr. Vargas did not provide information on the site of collection or a voucher specimen for this plant, making absolute identification and recollection difficult. Mr. Vargas indicated that PCN 98 was from the genus *Coccoloba* but he did not identify the species. Based on the characteristics of the collected fruits and stems of PCN 98 and a comparison with herbarium specimens of *Coccoloba* or other representatives of the Polygonaceae, it appears that PCN 98 was correctly identified as the genus *Coccoloba*. When contacted later about the identification, Mr. Vargas indicated that he believes he collected *Coccoloba tuerkheimii*, a newly identified species of *Coccoloba* from Costa Rica. However, a comparison of the morphological characteristics of the fruits from a herbarium specimen of *C. tuerckheimii* with the fruits of PCN 98 did not match, suggesting that PCN 98 was incorrectly identified.

Changes in the laws of Costa Rica relating to plant collection prohibited Mr. Vargas from recollecting this material or providing voucher specimens for conclusive identification. These regulations have placed restrictions on our attempts to recollect this and other species of *Coccoloba*. Although *Coccoloba* is found in the U.S., none of the tropical

botanical gardens has *C. tuerckheimii* or other Costa Rican species of *Coccoloba* in their collection.

A review of the literature indicates that there is enormous variation in morphology among the species of *Coccoloba*. According to Richard A. Howard's survey, there are differences in the juvenile and adventitious leaves of the same species of *Coccoloba*. He has also found differences between the leaves of juvenile species and flowering species. Results of his survey indicated that there are differences between the staminate flowering plants and pistillate flowering plants of the same species. According to Howard (1992), seven Coccoloba species from Mexico and Central America were originally identified by Standly (1928) based on characteristics of leaves from sterile specimens (Howard, 1992). In at least three of these species, specimens were taken from juvenile plants and not from adventitious shoots. Difficulty in matching recent fertile collections with the original specimens has arisen because of the enormous variation between collected specimens.

Howard also indicated that several species of *Coccoloba* from Panama originally identified as *C. acuplcensis* were incorrectly identified because of the natural variability among and within the species. Careful investigation by Howard revealed that these species belong to a new species from Panama

named *C. janstonii*. Another example of misidentification due to variation within the species was noted in the case of *C. lancifolia*. Howard's investigation proved that *C. lancifolia* was not a separate species but it is a Central American representative of the widespread Caribbean species, *C. diversifolia*. In his review, Howard suggested another collection of mature specimens of a *Coccoloba* species from Guatemala known as *C. cumbreance* will also be proven to be *C. diversifolia*.

Certainly, the possiblility exists that the person who collected the *Coccoloba* sp. in question misidentified the species. Fruit of PCN 98 resembled *C. uvifera* fruits collected from West Palm Beach, Florida. The *C. uvifera* fruits are slightly pear shaped and grow in grape-like elongated clusters. These fruits are burgundy in color when ripe and contain one seed in each fruit. This matches the exact description of the *Coccoloba* fruits (PCN 98) that were extracted for the antifungal assay. According to the Manual de la Flora de Costa Rica, *C. uvifera* is listed as one of the species of Coccoloba found in the Provincia de Limon in Costa Rica. Although the description of the fruit of PCN 98 fruit matches the description of *C. uvifera* fruit, additional information and recollection of PCN 98 will be required. This information will be necessary to confirm that the species of *Coccoloba*

(PCN 98) collected in the lowland rainforests of Costa Rica is a Costa Rican representative of *C. uvifera.* Because of the restrictions on the recollection of this species, further comparison between the *Coccoloba uvifera* from Florida and *Coccoloba* sp. (PCN 98) from Costa Rica was not possible. Therefore, absolute identification of the *Coccoloba* sp. collected from Costa Rica remains unresolved.

To date there have been no reports on the presence of antifungal activity in Coccoloba fruit. According to Neelis, *Coccoloba uvifera* has been used in traditional medicine to treat wounds, eruptions, rashes and hemorrhoids (Neelis, 1994). This investigator also mentioned that the astringent root, bark, and leaves of *C. uvifera* have been used to treat asthma, hemorrhage and diarrhea (Neelis, 1994). Fruits of *C. uvifera* are non-poisonous to humans, and ripe fruits are used to make jelly and wine-like alcoholic beverages. According to Malathi and co-workers *Coccoloba* species are used as a traditional medicine in Venezuela to treat tumors (Malathi et al., 1995). The same study also reported isolation of several secondary metabolites from the dried leaves of *C. uvifera* collected in Madras, India.

There has been only one report that suggests *Coccoloba* may have factors that deter insect feeding. Rodrigues and Saches demonstrated that *C. barbadensis* possessed some anti-insect activity. According to this study, when the vegetative portion of the *C. barbadensis* plants was dried, ground, and fed to *Sitophilus zeamais* larvae, the plant material caused 20% mortality of the insects (Rodriguez and Sanchez, 1994).

As far as it is known, this work is the first report on the presence of antifungal activity in the fruits of *Coccoloba* sp. (PCN 98). The antifungal activity extracted from *Coccoloba* fruits was heat and acid stable. The aqueous extract exhibited a broad range of activity against different pathogenic and saprophytic fungi. Inclusion of these extracts in the fungal growth assay caused total inhibition of growth of all fungi tested. The activity was retained after dialysis using 3,500 Da, 6-8000 Da and 12-14000 Da MWCO membranes. These results suggest that the active component is a macromolecule, possibly a protein with a molecular size greater than 14,000 Da.

Although the activity was retained after dialysis, there was no clear evidence of a single protein band on SDS-PAGE gels that corresponded to the activity. It is possible that the activity may not be a macromolecule (protein) but may be a low molecular weight peptide or other compound.

These components may aggregate forming larger complexes or may bind to higher molecular weight compounds. In this way, these complexes would be retained even after dialysis using membranes with a cut off limit of 12-14 kDa. The fact that no single protein band on the SDS gels corresponded with the antifungal activity may indicate that there is more than one active component in these extracts.

Heat and acid stability of the activity does not exclude the possibility that the active molecule is a protein or a polypeptide. The occurrence of heat and acid stable proteins and peptides in plants is not uncommon (see Chapter II). An acid and heat stable antifungal protein was purified by Broekaert and co-workers (Broekaert et al., 1989; Peumans et al., 1983) from rhizomes of *Urtica dioica*. This chitin-binding antifungal lectin was stable in 1 N HCl, 0.1 N acetic acid, 5% trichloroacetic acid and was still active after boiling. Antifungal defensins from *Amaranthus caudatus, Capsicum annum* and *Briza maxima* possess chitin-binding domain and are stable at extremes of pH. These defensins are unaffected by protease treatment and the antifungal activity is stable after boiling for 10 min (Broekaert, 1996). The antifungal activity purified from aqueous extracts of *Coccoloba* binds to chitin, was stable in 1 N HCl, 0.1 N acetic acid, 0.1 NaOH and was not affected by boiling for 5 min. The activity that eluted from the chitin-

column with 0.1 N NaOH was precipitated by ammonium sulfate again supporting the conclusion that the active component may be a protein.

When crude extract was fractionated by size-exclusion chromatography, the activity did not elute as a sharp peak but eluted as a broad band of activity. In the case of both the P-10 and S-200 columns, activity came off gradually as a broad peak. This broad diffuse zones of activity from these columns suggests that the active component interacts with the gel filtration resin. It is not uncommon for proteins to bind to various gel filtration media through ionic, hydrophobic or affinity (ligand-protein) interactions. In fact, various resins such as Sepharose have been used effectively to isolate lectins and other carbohydrate-binding proteins. Originally, it was assumed that the active component was a macromolecule and the pigmented portion of the extract was a low molecular weight compound. To separate the active fraction from the pigmented fraction the crude dialyzed extract was fractionated on a Biogel P-10 column. The latter has a molecular weight exclusion limit of about 10,000 Da. Based on this hypothesis, we would predict that the larger active molecule will come off along on the void volume of the column and the pigmented portion will come off later due to its smaller size. In fact, the active molecule eluted as a

broad peak from the void volume to the column bed volume. Based on this result, the possibility exists that the active component, or components, varies in size from low molecular weight to greater than 10,000 Da. Another possible explanation is that the active molecule interacts with the column matrix through hydrophobic, ionic or other interactions.

The pigmented portion did not elute with buffer but eluted with 0.1 M NaOH. This result suggests that the pigmented material was bound tightly to the column through hydrophobic interactions and this interaction was disrupted in the presence of 0.1 M NaOH. We propose that the active material may interact with the pigmented material through hydrophobic bonds and that this association is not as strong as the interaction between the resin and the pigmented substance. Thus, the active material slowly dissociates from the pigmented-column complex during chromatography with buffer elution. This slow release results in a broad band of activity being eluted from the column. The pigmented substances remain bound to the column until these hydrophobic bonds are disrupted by strong base. With respect to size, one would predict that the active material has a molecular weight of around 10,000 Da or larger. Based on this analysis, one

can not predict the size of the pigmented material. Overall, aggregate complex between the active component(s) and the pigmented substances have an effective size greater than 12-14,000 Da and, therefore, these complexes are retained during dialysis. This interpretation is consistent with the results obtained with preparative isoelectric focusing. In this instance, the glycerol which enhances/stabilizes hydrophobic interactions caused the pigmented material to aggregate and precipitate. Since there was no antifungal activity detected in any of the Rotofor fractions, we proposed that the antifungal component remained associated with the pigment and both were precipitated.

Very little information was gained from preparative IEF. When IEF was carried out on acrylamide gels, the only protein bands detected in the active fractions appeared to be acidic. This result is consistent with the results obtained from anion and cation-exchange chromatography. The active component remained tightly bound to an anion-exchange column at pH 8.0 and did not elute even with 2 M NaCl. This suggests that the active component was highly negatively charged at pH 8.0 (i. e. the pI must be much lower than pH 8.0). In support of this conclusion, the active fraction

did not bind to a cation-exchange column and came off in the flow through (i.e. activity was not positively charged). By lowering the pH of the elution buffer to pH 6.0, some activity came off the column in the flow through, i. e. pI must be between 6 and 8. Some activity was bound and eluted with the salt gradient, i. e. this component must have a pI below 6. The extract applied to the column still contained pigmented substances and it is possible that these compounds were bound to the column and affected elution. Alternatively, the active component may have separated from the pigment during IEF but may have become insoluble and lost activity at it's pI.

More work is needed to further purify the antifungal activity from extracts of *Coccoloba* (PCN 98) fruit. In the future, it would be necessary to determine whether lower molecular weight polypeptides are present which correlate with the activity. This can be addressed by using high-resolution peptide gels. One needs to determine whether there is more than one active component. Results to date suggest that this may be the case. Based on analogy with heat and acid stable chitin-binding peptides, the active material may be a lectin. To test this hypothesis hemagglutination assays should be performed.

Although the work we report on herein is preliminary, the results are extremely promising once more material can be collected. This will require

confirmation of the species identity and/or studies with ripe fruits of *Coccoloba uvifera* from Florida. Promising approaches to purification include a combination of ion-exchange chromatography (different pH's, anion and/or cation-exchange chromatography), affinity chromatography using a chitin column or sugar/lectin affinity columns and P-10 to separate the active material from the pigmented substances.

CONCLUSIONS

In conclusion, these results are extremely promising. Fruits of *Coccoloba* (PCN 98) contain one or more potent inhibitors of fungal growth. Our results are consistent with the suggestion that the inhibitory substance(s) may be a polypeptide or protein. The future work will rely on the correct identification of the *Coccoloba* sp. and collection of more fruits of the identified species.

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