Biochemical and Biological Characterization of Lectins, Hemagglutinin and Antifungal Proteins from Seeds

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I declare that the assignment here submitted is original except for source material explicitly acknowledged, and that the same or related material has not been previously submitted for another course. I also acknowledge that I am aware of University policy and regulations on honesty in academic work, and of the disciplinary guidelines and procedures applicable to breaches of such policy and regulations, as contained in the website.

Signature	Date
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List of Publications

Lam SK, Ng TB. (2009) A dimeric high-molecular-weight chymotrypsin inhibitor with antitumor and HIV-1 reverse transcriptase inhibitory activities from seeds of *Acacia confusa*. Phytomedicine 17: 621-625.

Lam SK, Ng TB. (2009) Acafusin, a dimeric antifungal protein from *Acacia confusa* seeds. Protein Pept Lett Epub.

Lam SK, Ng TB. (2009) Novel galactonic acid-binding hexameric lectin from *Hibiscus mutabilis* seeds with antiproliferative and potent HIV-1 reverse transcriptase inhibitory activities. Acta Biochim Pol 56: 649-654.

Lam SK, Ng TB. (2010) Isolation and characterization of a French bean hemagglutinin with antitumor, antifungal, and anti-HIV-1 reverse transcriptase activities and an exceptionally high yield. Phytomedicine 17: 457-462.

Lam SK, Ng TB. (2010) First report of a hemagglutinin-induced apoptotic pathway in breast cancer cells. Biosci Rep 30: 307-317.

Lam SK, Ng TB. (2010) First report of an antifungal amidase from *Peltophorum ptercoarpum*. Biomed Chromatogr 24: 458-464.

Lam SK, Ng TB. (2009) Passiflin, a novel dimeric antifungal protein from seeds of the passion fruit. Phytomedicine 16: 172-180.

Lam SK, Ng TB. (2009) A protein with antiproliferative, antifungal and HIV-1 reverse transcriptase inhibitory activities from caper (*Capparis spinosa*) seeds. Phytomedicine 16: 444-450.

Lam SK, Han QF, Ng TB. (2009) Isolation and characterization of a lectin with potentially exploitable activities from caper (*Capparis spinosa*) seeds. Biosci Rep 29: 293-299.

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Abstract of thesis entitled: Biochemical and biological characterization of lectins, hemagglutinin and antifungal proteins from seeds

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Abstract

The seeds contain an abundance of proteins, some of which are storage proteins but may play a role of protection from pathogenic microbes and phytophagous insects. Antifungal peptides/proteins, antiviral proteins, ribosome-inactivating proteins, proteinase inhibitors, chitinases, proteinases, and defensins, are some examples of the myriad of seed proteins. The aforementioned proteins are collectively called plant defense proteins in view of their antipathogenic activities. These antifungal proteins exhibit a wide range of molecular masses and amino acid sequences.

Lectins and hemagglutinins are carbohydrate binding proteins present in a diversity of organisms including humans, vertebrate and invertebrate animals, plants, fungi, and bacteria. They are usually the abundant storage proteins in leguminous plants. They display a host of biological activities such as antitumor, antifungal, antiviral,

insecticidal, and antibacterial activities.

Two lectins with potentially exploitable activities were purified from *Capparis spinosa* seeds and *Hibiscus mutabilis* seeds, respectively. A hemagglutinin was isolated from *Phaselous vulgaris*, cultivar "French bean 35", and detailed apoptotic pathway in breast cancer cells, MCF-7 cells, was investigated. A novel dimeric β-lactoglobulin-like antifungal protein and an antifungal amidase were purified from *Passiflora edilus* seeds and *Peltophorum pterocarpum*, respectively.

The biological properties of isolated proteins, including hemagglutinating, antifungal, anti-tumor and HIV-1 reverse transcriptase inhibitory activities, were examined. Their biochemical and biological properties were compared with other purified proteins.

論文摘要

種子含有豐富的蛋白質，其中一部份作貯藏用途，也有一些可起到抗病原，抗微生物和抗植食性昆蟲的保護作用。鑑於其抗病特性，它們統稱爲防禦蛋白，例子包括抗真菌多肽/蛋白質，抗病毒蛋白，核糖體失活蛋白，蛋白酶抑製劑，幾丁質酶，蛋白酶和防禦素。它們擁有不同的分子量和氨基酸序列。

外源凝集素和紅血球凝聚素是碳水化合物結合蛋白，目前已在不同生物中提純，包括人類，脊椎和無脊椎動物，植物，真菌和細菌。它們在儲豆科植物中的含量十分是豐富，並擁有不同的生物活性，如抗腫瘤活性，抗真菌活性，抗病毒活性，殺蟲活性和抗菌活性。

我們提純了老鼠瓜(*Capparis spinosa*)種子的外源凝集素，木芙蓉(*Hibiscus mutabilis*)種子的外源凝集素，肉豆 35(*Phaselous vulgaris*, cv "French bean 35")的紅血球凝聚素，熱情果(*Passiflora edilus*)的新型的類 β-乳球蛋白類抗真菌蛋白，和雙翼豆(*Peltophorum pterocarpum*)的抗真菌酰胺酶。在提純完畢後，我們研究了它們不同的生物活性，包括血凝能力，抗真菌能力，抗腫瘤能力和 HIV-1 逆轉錄酶抑制能力，和進行了肉豆 35 的紅血球凝聚素在乳腺癌細胞 MCF-7 細胞中凋亡途徑的詳細研究，並與其他已純化的蛋白質進行比較。

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List of Abbreviations

µl	Microliter
CIM	Convective Interaction Media
ConA	Concanavalin A
DEAE-cellulose	Diethylaminoethyl-cellulose
DIG	Digoxigenin
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
FITC	Fluorescein Isothiocyanate
ha	Hemagglutinating Activity
HIV	Human Immunodeficiency Virus
HRP	Horseradish Peroxidase
IC_{50}	Half Maximal Inhibitory Concentration
IgG	Immunoglobulin G
JC-1	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide
kDa	Kilodalton
MES	2-(N-morpholino)ethanesulfonic Acid
min	Minute
ml	Milliliter
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium Bromide
ng	Nanogram
^{o}C	Degree Celsius
PBS	Phosphate Buffered Saline
PI	Propidium iodide
PMSF	Phenylmethanesulfonylfluoride
POD	Peroxidase
PVDF	Polyvinylidene Fluoride
RIP	Ribosome-Inactivating Protein
RNase	Ribonuclease
RT	Reverse Transcriptase
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis
SP-Sepharose	Sulfopropyl-Sephaorse
TBS-T	Tris-buffered saline with 0.1% Tween 20
TLP	Thaumatin-Like Protein
Tris	Tris(hydroxymethyl)aminomethane

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General Introduction

Plants are attacked by different organisms, including bacteria, viruses, fungi, insects, and herbivores. Therefore, they have developed different defense mechanisms, such as accumulation of phytoalexins, localized cell death, oxidative burst and production of cell wall strengthening proteins. On the other hand, they also produce different kinds of plant defense proteins, e.g. lectins and hemagglutinins, ribonucleases, ribosome-inactivating proteins and different types of antifungal proteins.

Lectins and hemagglutinins were extensively reviewed in this study. Other plant defense proteins, including chitinases, ribonucleases, ribosome-inactivating proteins, thaumatin-like proteins, and protease inhibitors, were discussed briefly. Further examples are proteases, defensins, thionins, cyclophilin-like proteins, killer proteins, hemolysins, etc. There are still many proteins with novel N-terminal sequences that display antifungal activity. They are generally called antifungal proteins.

By understanding the breadth of induced defense responses and the mechanisms employed to control these pathways, genetic engineering for genes of insecticidal proteins, protease inhibitors, antifungal proteins, and plant lectins, etc, can be used to limit damage done by insects and fungal infections. Novel crop protection strategies based on genetic engineering show great potential to reduce crops damage by

pathogens. Furthermore, their anti-viral and anti-tumor properties made them possible for anti-viral and anti-tumor drugs.

So, the identification and characterization of different plant defense proteins, including lectins, hemagglutinin and antifungal proteins, were focused on this study.

Chapter 1: Literature Review of Lectins, Hemagglutinin and Antifungal Proteins

1.1. History and Overview of Lectins

"Lectin" comes from the Latin word "legere", which means "to select", by William Boyd in 1954. Lectins have the ability to bind carbohydrates. In 1860, Mitchell demonstrated agglutination activity between rattlesnake venom lectin and pigeon blood. On the other hand, Elfstrand firstly introduced the term "hemagglutinin" as the substances with hemagglutinating activity in 1898. Nowadays, proteins that can agglutinate blood cells with known sugar specificity are referred to as "lectins". If the sugar specificity is unknown, they are called "hemagglutinins".

Lectins and hemagglutinins are proteins/glycoproteins, which have at least one non-catalytic domain that binds reversibly to specific monosaccharide or oligosaccharide. They can agglutinate erythrocytes by binding to the carbohydrate moieties on the surface of erythrocytes, without changing the properties of the carbohydrates. As a result, enzymes that substitute group into carbohydrates are not lectins, e.g. glycosidases and glycosytransferases.

The most famous lectin is concanavalin A (Con A). It was purified from *Canavalia ensiformis* (Jack bean) (Summer, 1919). It is also the first lectin to be isolated massively and commercially available. There is no blood group specificity

for Con A. The agglutinating activity of Con A can be inhibited by α-D- mannosyl and α-D-glucosyl residues. Calcium ions and manganese ions are essential for its activity. Con A is a tetramer when pH is from 5.8 to 7.0, and forms aggregates when pH is higher than 7. It is used for characterization and isolation of cellular structures and sugar-containing molecules. The crystallographic structure of Con A is shown in Figure. 1.1. (Hardman and Ainsworth, 1972).

1.1.1. Distribution of Lectins

Lectins have been purified from various parts of plants, including flowers (Flower et al., 1984), fruits (Arslan and Chulavatnatol, 2000), stems (Biswas et al., 2009), leaves (Singh et al., 2006), and bulbs (Kakehi et al., 2003). They are mostly found in seeds (Echemendia-Blanco et al., 2009), especially from legume seeds (Sharma et al., 2009).

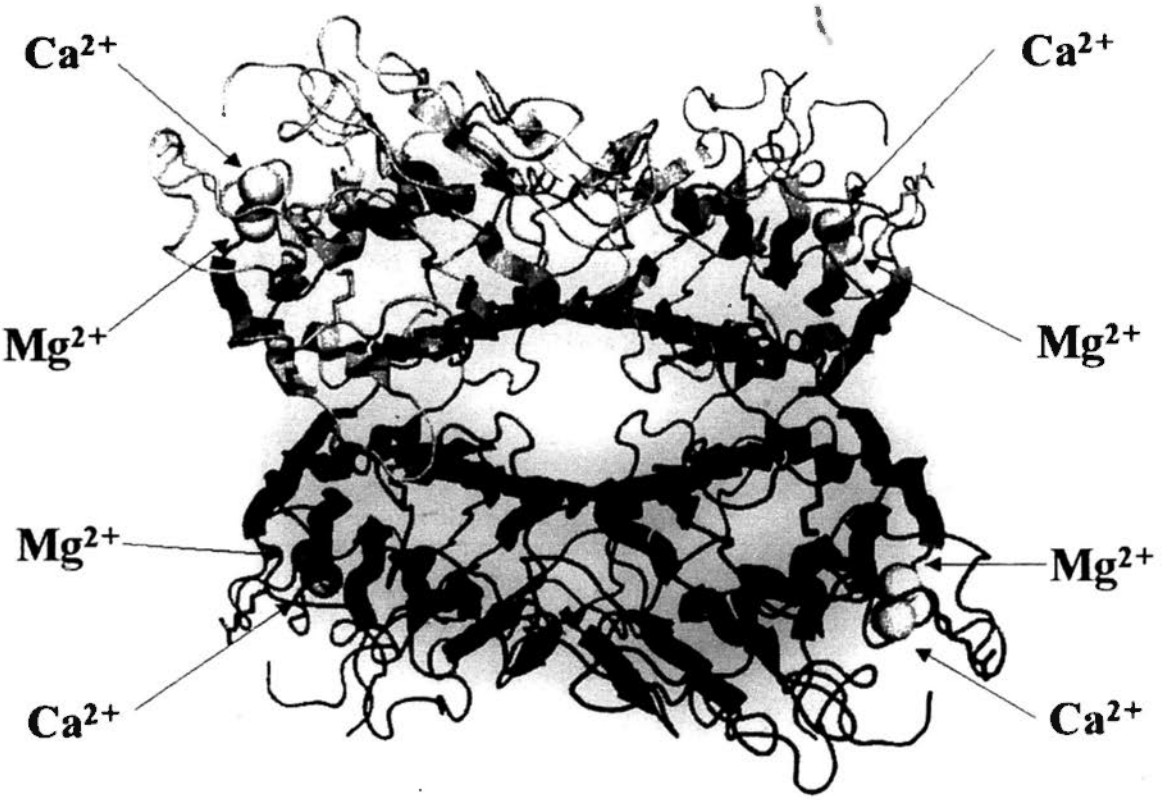


Figure 1.1. Crystallographic structure of a tetramer of jack bean concanavalin A (Hardman and Ainsworth, 1972).

1.1.2. Classification of Lectins

1.1.2.1. Classification of Lectins According to Carbohydrates Specificity

Lectins have been purified from different organisms. They all have specific carbohydrate specificity. Therefore, the classification of lectins can be based to their carbohydrate specificity. They include glucose/mannose specific (Naeem et al., 2007), fucose specific (Argayosa and Lee, 2009), galactose specific (Cao et al., 2009), N-acetylglucosamine specific (Adhya et al., 2009), mannose specific (Ohizumi et al., 2009) and sialic acid (N-acetylneuraminic acid) specific (Chen et al., 2009). Examples of different carbohydrate specificity are listed in Table 1.1. Later on, lectins with other carbohydrate specificity were purified.

However, some lectins cannot be classified according to such a system. An arabinose-specific lectin was isolated from *Peziza sylvestris* (Wang and Ng, 2005b). An inulin-specific lectin was purified from *Xerocomus spadiceus* (Liu et al., 2004). Some lectins can be inhibited by more than 3 sugars. *Pleurotus citrinopileatus* lectin can be inhibited by o/p-nitrophenyl-β-d-glucuronide, o/p-nitrophenyl-beta-d-galactopyranoside, maltose and inulin (Li et al., 2008). Seven carbohydrates, including melibiose, lactose, D-galactose, alpha-methyl-D-galactopyranoside, N-acetylneuraminic acid, raffinose and inulin, could inhibit the hemagglutinating activity of *Agrocybe cylindracea* lectin (Wang et al., 2000).

Table 1.1. Examples of lectins with different carbohydrate specificities.

Carbohydrate specificity of lectins	Source of lectins
Glucose/mannose	*Trigonella foenumgraecum* (Naeem et al., 2007) Emperor banana (Wong and Ng, 2006) *Annona muricata* (Damico et al., 2003) *Pisum arvense* (Cavada et al., 2003) *Cajanus cajan* (Naeem et al., 2001)
Fucose	*Neoditrema ransonnetii* (Nakamura et al., 2009) *Aristichthys nobilis* (Pan et al., 2009) *Oreochromis niloticus* (Argayosa and Lee, 2009) Snails (Mansour and Abdul-Salam, 2009) *Lucioperca lucioperca* (Antoniuk, 2004)
Galactose	*Musca domestica pupae* (Cao et al., 2009) *Aplysia kurodai* (Kawsar et al., 2009a) *Trichosanthes cordata* (Sultan et al., 2009) *Perinereis nuntia* (Kawsar et al., 2009b) Pinto bean (Wong and Ng, 2006)
N-acetylglucosamine	*Macoma birmanica* (Adhya et al., 2009) *Xenopus laevis* (Dehennaut et al., 2008) *Koelreuteria paniculata* (Macedo et al., 2003) *Oudemansiella platyphylla* (Matsumoto et al., 2001)
Mannose	*Dioscorea batatas* (Ohizumi et al., 2009) *Dendrobium findleyanum* (Sudmoon et al., 2008) *Acropora millepora* (Kvennefors et al., 2008) *Polygonatum cyrtonema hua* (Ding et al., 2008) *Sophora flavescens* (Liu et al., 2008a)

1.1.2.1. Classification of Lectins According to Overall Structures

Another system, according to the overall structures of lectins, can be used to classified lectin into merolectins, holoectins, chimerolectins and superlectins.

Merolectins are small monomeric lectins with only one carbohydrate-binding domain. They are devoid of agglutinating activity due to their monovalent nature. Hevein from *Hevea brasiliensis* (Van Parijs et al., 1991) and orchids monomeric-binding protein (Van Damme et al., 1994) are some examples.

Hololectins have two or more identical or similar carbohydrate-binding domains, so they have agglutinating activity (Vervecken et al., 2000). They can bind to the same or structural analogous carbohydrates. The most famous example is Con A purified from *Canavalia ensiformis* (Surolia et al., 1973). Phytohaemagglutinin from *Phaseolus vulgaris* is another example (Schumacher et al., 1971).

Chimerolectins are fusion proteins that make up of a peptide with carbohydrate-binding domain and other peptide with a non-carbohydrate-binding domain. One example is the type II RIP ricin (Ishiguro et al., 1964).

Superlectins are a type of chimerolectins with two tandemly arrayed carbohydrate-binding domains. It can bind to structurally unrelated carbohydrates. Tetrameric tulip bulb lectin exhibits a complex sugar specificity toward rabbit erythrocytes. Its agglutination activity toward human erythrocytes is inhibited by

N-acetylgalactosamine, lactose, fucose and galactose (Cammue et al., 1986). *Agrocybe cylindracea* lectin is another example in which the hemagglutinating activity could be inhibited by multiple carbohydrates (Wang et al., 2000).

1.1.2.3. Classification of Lectins According to Families

Apart from using carbohydrates specificity and structure to classify lectins, lectins can be grouped into different families, which are legume lectins, type II RIPs, monocot mannose-binding lectins and other lectins.

1.1.2.3.1. Legume Lectins

The legume lectins are a family of lectins found in the Leguminosae family. The lectin content in the seeds is very high, while the content is much lower in the bark, leaves, roots and stems of leguminous plants. Leguminoseae is the third largest family of flowering plants that can be found throughout the world. Its common names are the bean family, legume family, pulse family, or pea family. Legume is the fruit/seed of Leguminosae. Leguminosae, plays an important role economically, has been cultured throughout the world, and serve as essential sources of food. *Arachis hypogaea* (peanut), *Cicer arietinum* (chickpeas), *Glycine max* (soybean), *Medicago sativa* (alfalfa), *Phaseolus* (beans), *Pisum sativum* (pea) are the most common known members of Leguminosae.

More than 100 lectins have been isolated from leguminous plants. They have

different carbohydrate specificities, for instance, galactose-specific lectin from *Phaseolus vulgaris* cv. extralong autumn purple bean (Fang et al., 2010) and melibiose-specific lectin from Chinese black soybeans (Lin et al., 2008).

They are usually dimeric or tetrameric, with subunit molecular weight from 25 kDa to 32 kDa. Each subunit contains a carbohydrate-binding site. Divalent metals (e.g. Ca^{2+} and Mn^{2+}) are essential for their agglutinating activity. A significant number of invariant amino acid residues are located in metal binding sites. The three-dimensional structures of different legume lectins have disclosed that the locations of the metal and carbohydrate-binding sites of the legume lectins are similar (Sharon and Lis, 1990).

1.1.2.3.2. Type II Ribosome-Inactivating Proteins (RIPs)

Type II RIPs have both A chain (N-glycosidases) and B chain (galactose-specific lectin), linked by disulfide bond. Ricin is possibly the best well-known member of type II RIPs (Endo et al., 1987). The structure of ricin is displayed in Figure 1.2. (Weston et al., 1994). Mistletoe (*Viscum album*) lectins (Ye et al., 2006), aralin from *Aralia elata* (Tomatsu et al., 2004), cinnamomin from camphor tree (Xu and Liu, 2004), ebulin l from the leaves of *Sambucus ebulus* L (Pascal et al., 2001), sieboldin-b from the bark tissue of Japanese elderberry (Rojo et al., 1997) are some examples of type II RIPs.

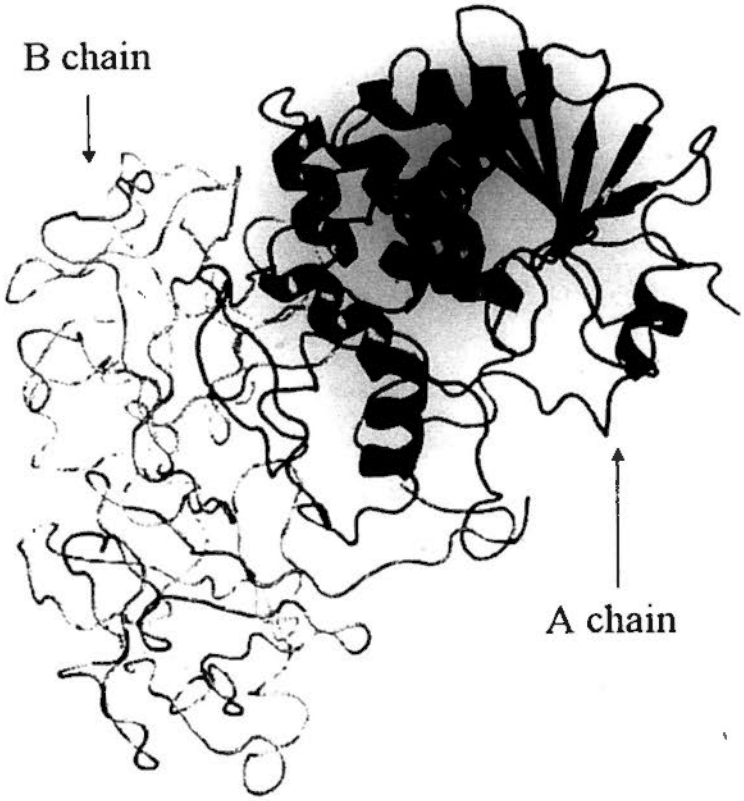


Figure 1.2. Structure of ricin (Weston et al., 1994).

1.1.2.3.3. Monocot Mannose-Binding Lectins

Listera ovata lectin is the first monocot mannose-binding lectin reported, which displays a high affinity toward with mannose (Van Damme et al., 1987). It is different from other mannose/glucose-specific lectins from dicots such as concanavalin A, and pea lectin. Mannose-binding lectins have been purified and characterized from Amaryllidaceae, Alliaceae, Araceae, Liliaceae, Orchidaceae, and Iridaceae families (Van Damme et al., 2000). There are three carbohydrate-binding motifs per subunit with a consensus sequence signature QXDXNXVXY, which is critical for mannose binding (Balzarini et al., 1991).

Monocot mannose-binding lectins are hololectins composed of two or four identical one-domain promoters. The carbohydrate specificities of lectins depend on oligomerization and multivalency. For example, snowdrop tetrameric monocot mannose-binding lectin can bind with mannan epitopes on GP120, the major glycoprotein of human immunodeficiency virus (Balzarini et al., 1991).

1.1.2.3.4. Other lectins

Phloem lectins and jacalin are lectins with amino acid sequences different from legume lectins, type II RIPs or monocot mannose-binding lectins.

The alpha-chains of the *Moraceae* lectins, jacalin and *Maclura pomifera* agglutinin are 133 residues long with 85% homology. They have a conserved

tryptophan residue, which may be involved in the binding site (Young et al., 1991).

Phloem lectins are located in the phloem sap, which is translocated in the assimilate stream where its lectin activity can exert effects over long distances. The genes of phloem lectins can be found in 17 angiosperm and gymnosperm genera. The molecular mass of phloem lectin is varied, but these lectins are composed of a highly conserved domain (Dinant et al., 2003).

1.1.3. Physiological Roles of Plant Lectins and Their Applications

1.1.3.1. Lectins as Defense Proteins

Some lectins show anti-insect activity. When *Bactrocera cucurbitae* (Coquillett) was fed with *Arisaema jacquemontii Blume* lectin, the development of its larvae was retarded with a significant decrease in acid phosphatase and alkaline phosphatase activities while esterase activity was markedly increased (Kaur et al., 2006a). *Arisaema helleborifolium Schott* lectin also showed anti-insect activity towards larvae of *Bactrocera cucurbitae* (Coquillett) with the same effects (Kaur et al., 2006b). The feeding of *Bauhinia monandra* leaf lectin in an artificial diet could produce 50% mortality to cowpea weevil and Mexican bean weevil, while the weight of Mediterranean flour moth larvae was decreased by 40 % (Macedo et al., 2007). Therefore, lectins are useful in a biotechnological strategy for insect management and development of insect resistance in important agricultural crops. A recent success

example is the introduction of the coding sequence of *Allium sativum* leaf agglutinin in a rice cultivar to obtain sustainable protection from attack of sap-sucking planthoppers (Sengupta et al., 2010).

Among thousands of lectin and hemagglutinin purified, only a few of them possess antifungal activity (Table 1.2). The expression of *Gastrodia elata* lectins in the vascular cells in roots and stems was strongly induced by the fungus *Trichoderma viride* indicating that lectin is an important plant defense protein (Sa et al., 2003). The precursor gene of stinging nettle isolectin I was inserted into tobacco and the germinated spores of the *Botrytis cinerea*, *Colletotrichum lindemuthianum*, and *Trichoderma viride* were significantly reduced (Does et al., 1999). So, lectins may be introduced into plants to prevent them from fungal attack.

An experiment showed that mice treated with a very high dose of tepary bean lectin had severe intestinal inflammation, thymus degeneration, and a significant increased spleen weight. The treatment would also cause damage in lungs, kidneys, heart, liver and thymus, and even death (Reynoso-Camacho et al., 2003). Thus, the application of lectins should be investigated carefully and under control.

Table 1.2. Examples of lectins with antifungal activity.

Source of lectin	Fungal species inhibited	Reference
Curcuma amarissima Roscoe	*Fusarium oxysporum, Exserohilum turicicum, Colectrotrichum cassiicola*	Kheeree et al., 2009
Talisia esculenta	*Microsporum canis*	Pinheiro et al., 2009
Maize	*Aspergillus flavus*	Baker et al., 2009
Phaseolus coccineus (*Phaseolus multiflorus willd*)	*Helminthosporium maydis, Sclerotinia sclerotiorum, Gibberalla sanbinetti, Rhizoctonia solani*	Chen et al., 2009
Red cluster pepper (*Capsicum frutescens*)	*Aspergillus flavus, Fusarium moniliforme*	Ngai and Ng, 2007a
Amaranthus viridis Linn	*Botrytis cincerea, Fusarium oxysporum*	Kaur et al., 2006c
Phaseolus vulgaris cv red kidney bean	*Fusarium oxysporum, Coprinus comatus, Rhizoctonia solani*	Ye et al., 2001

1.1.3.2. Lectins as Storage Proteins

Lectins are typically found in storage vacuoles, extracellular compartment, cytoplasm and the nucleus. They are abundant in seeds, especially legume seeds. Japanese chestnut agglutinin mRNA was expressed in the stems, flowers and seeds with a high level during development and germination. So, it may play a role as storage protein (Nakamura et al., 2004). The mannose-binding lectin is also a tuber storage protein (Shewry et al., 2003). As mentioned before, lectins are also plant defense proteins. When plants are attacked by bacteria, fungi, insects or animals, lectins are released from vacuoles, extracellular compartment, cytoplasm and the nucleus, so as to protect the plants.

1.1.3.3. Nitrogen Fixation Capability of Lectins

There is a symbiotic relationship between leguminous plants and nitrogen-fixing bacteria. Wheat germ agglutinin can bind to the agglutinin-binding receptor on the cell membrane of *Azospirillum lipoferum*, which is then stimulated to elevate transcription of the nitrogenase enzyme. As a result, the signaling cascade is triggered and nitrogen fixation capability is increased (Karpati et al., 1999). Lectins of the nitrogen-fixing *Paenibacilli* can increase the rate of degradation of cellulose in the plant cell, thus enhancing β-glucosidase activity in the wheat-root cell wall. As a result, the rate of nitrogen fixation is increased (Karpunina et al., 2003).

1.1.4. Biological Roles of Lectins and Their Applications

1.1.4.1. Immunomodulatory Activity

Galectins are found in different kind of cells of the immune system. They modulate immune responses including T cell survival, activation, differentiation, and cytokine secretion. They are involved in cell activation, differentiation, and apoptosis of T cells. They play important roles in the modulation of chronic inflammatory disorders, autoimmunity, and cancer (Ilarregui et al., 2005).

Pro-inflammatory and anti-inflammatory mediators are produced during inflammation. Galectins act as pro-inflammatory or anti-inflammatory agents of acute and chronic inflammatory responses. They play important roles in chronic inflammation and participate in the homeostasis of the inflammatory response. They can regulate cell survival and signaling, influence cell growth and chemotaxis, interfere with cytokine secretion, mediate cell-cell and cell-matrix interactions (Rabinovich et al., 2002). Galectins are also involved in regulating cell adhesion, migration, chemotaxis, antigen presentation, immune cell activation and apoptosis (Rubinstein et al., 2004).

1.1.4.2. Anti-tumor Activity

Reaction between leukemic cell surfaces and wheat germ agglutinin has been observed (Aub et al., 1965). Similar interaction between soybean agglutinin and

animal cell surfaces was found (Lis et al., 1970). The investigations of lectins and tumor cells were then carried out. *Ricinus communis* lectin showed inhibition of tumor cells (Gürtler and Steinhoff, 1972).

Later on, the study of anti-tumor mechanisms was started. Inhibition of DNA synthesis and cellular metabolism was induced by phytohemagglutinin in human leukemic cells (Borrebaeck and Schön, 1987). Mistletoe lectins displayed cytotoxic effect in different tumor cell lines. Membrane perforation and protusions were observed. Cytokines were released. Tumor growth was suppressed *in vivo*. The anti-tumor efficacy of tumor necrosis factor α is enhanced by lectins. An anti-angiogenic response in the host was induced by lectins. The anti-tumor effect was due to an inhibition of tumor-induced angiogenesis and an induction of apoptosis (Pryme et al., 2006).

The intensive study of action mechanisms in tumor cell lines and animal model studies make the use of lectins in cancer therapy feasible.

1.1.4.3. Antiviral Activity of Lectins

The *Gerardia savaglia* D-mannose-specific lectin was firstly reported to prevent infection of H9 cells with HIV-1. Furthermore, the lectin inhibited syncytium formation in the HTLV-IIIB/H9-Jurkat cell system and HIV-1/human lymphocyte system by reacting with the oligosaccharide side chains of the HIV-1 gp120 envelop

molecule (high-mannose oligosaccharides) (Müller et al., 1988). One year later, the lectins concanavalin A, wheat germ agglutinin, *Lens culinaris* agglutinin, *Vicia faba* agglutinin, *Pisum sativum* agglutinin and phytohaem(erythro)agglutinin were found to bind to gp120. They were able to inhibit fusion of HIV-infected cells with CD4 cells by a carbohydrate-specific interaction with the HIV-infected cells (Hansen et al., 1989).

The treatment of AIDS with lectins was further investigated. Different lectins have different anti-HIV mechanisms. More recently, Polychaete marine worm *Chaetopterus variopedatus* lectin inhibited cytopathic effect induced by HIV-1 and the production of viral p24 antigen (Wang et al., 2006). The sea worm *Serpula vermicularis* lectin could inhibit the production of viral p24 antigen and cytopathic effect induced by HIV-1 (Molchanova et al., 2007). *Polygonatum cyrtonema Hua* lectin inhibited HIV-I- and HIV-II-induced cytopathicity in MT-4 and CEM cells (An et al., 2006). Banana lectin could directly bind the HIV-1 envelope protein (gp120) and blocked entry of the virus into the cell, and decreased the levels of the strong-stop product of early reverse transcription (Swanson et al., 2010). Extralong autumn purple bean lectin (Fang et al., 2010) and mushroom *Russula delica* lectin (Zhao et al., 2010) were able to inhibit the HIV-1 reverse transcriptase. Lectins are potential drugs for treatment of AIDS.

1.1.6. Other Applications

Lectin affinity chromatography is a form of chromatography in which lectins are immobilized on gel beads. Glycoproteins are then adsorbed by lectins and eluted with a specific carbohydrate. So, it can fractionate and purify glycoproteins based on their specific features. Moreover, serial lectin column chromatography is useful for isolation of extremely small amounts of glycoproteins. In combination with other separation techniques, oligosaccharides can be purified rapidly.

Eight lectin-Sepharose columns were used to separate different commercial types of recombinant human erythropoietin, erythropoietin analogues and urine human erythropoietin from healthy individuals (Franco Fraguas et al., 2008). Lectin affinity chromatography, such as immobilized Concanavalin A, was applied in the isolation of glycopeptides that express biantennary and hybrid N-linked structures and high mannose glycans, which are abundant in both embryonic stem cells and embroid bodies stages (Alvarez-Manilla et al., 2009).

The structures of glycoproteins are difficult to analyze because of the linked carbohydrate. Lectin microarray provides a solution. Panels of lectins are immobilized on a single chip in an array format. The fluorescent-tagged samples are hybridized to the array. After analysis of the spots binding pattern, carbohydrate composition information of the sample can be obtained, even the glycosylation of

samples is small (Figure 1.3) (Pilobello and Mahal, 2007). Most recently, lectin microarray was applied in distinguishing mammalian cells that infected with the intracellular apicomplexan parasite *Cryptosporidium parvum*. The cells were probed with fluorescent-labeled lectins. N-acetyl-D-galactosamine binding soybean agglutinin generated the largest signal difference due to the over-expression of glycoprotein on the infected cells surface and the glycoprotein located in the intracellular parasites (Yang et al., 2009).

The carbohydrate specificity of lectins is used to recognize samples. The steroid hapten digoxigenin-conjugated lectins enable immunological detection the structures of the bound lectins. Lectins specially identifying the terminal sugars are used, thus allowing the carbohydrate chain to be identified. It was used to modify the glycosylation patterns of cell surface glycoconjugates during thymocyte selection processes of postnatal days of murine (Balcan et al., 2008).

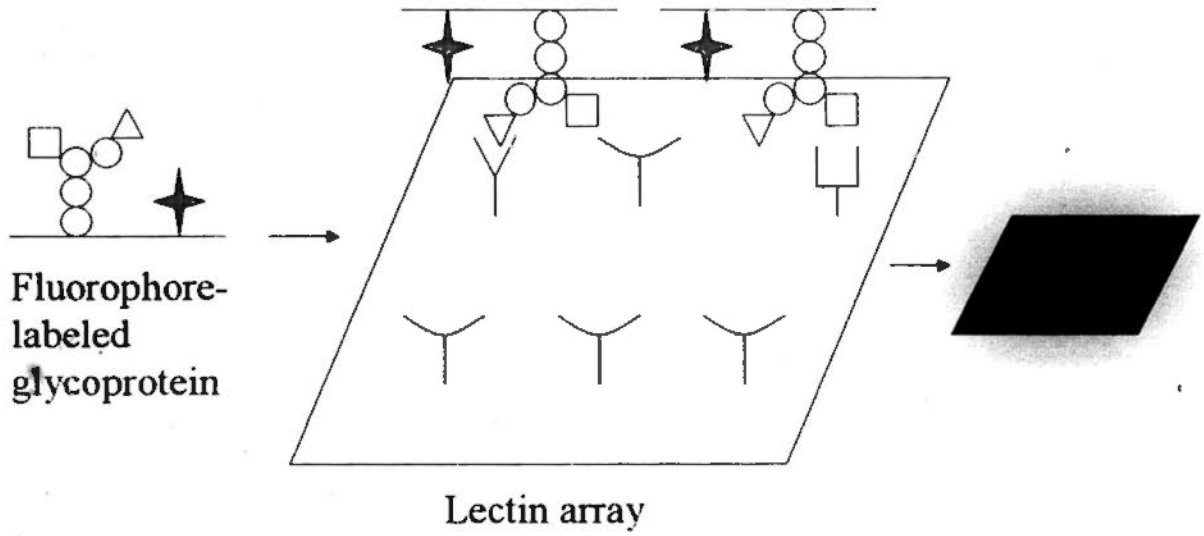


Figure 1.3. Lectin microarray. The fluoresecently-labeled cells or proteins are hybridized to the lectin array. The glycoslation of sample can be analyzed by the resulting pattern.

1.2. Other Plant Defense Proteins

Lectins and hemagglutinins are well-known members in plant defense proteins. There are many other plant defense proteins, which have antifungal, anti-viral or anti-bacterial activities, including chitinases, ribonucleases, ribosome-inactivating proteins, thaumatin-like proteins, and protease inhibitors. Other examples are proteases, defensins, thionins, cyclophilin-like proteins, killer proteins, hemolysins, etc.

1.2.1.1. Overview of Chitinases

Chitinases are omnipresent enzymes, found in animals, bacteria, fungi, and plants. The most recent purified chitinases are listed in Table 1.3. They hydrolyze the β-1,4-linkage between N-acetylglucosamine residues of chitin. The polysaccharide of the cell wall of many fungi (major pathogens) and exoskeletons of invertebrates (pests) are made of chitin.

The major application of chitinases is to increase the yield of crops by increasing the resistance of transgenic plants to pathogens. However, the quality of the products may be decreased, an inhibition or delay of hyphal extension in transgenic pea was observed (Hassan et al., 2009).

The classification of chitinases, based on the presence or absence of chitin-binding domain and the sequence homology, can be classified into family 19,

family 18 and PR-4 family chitinases

Chitinases are endo-β-1,4-glucosaminidases, which can hydrolyze the β-glycosidic bond at the reducing end of glucosamidines. Chitin is a β-1,4-polymer of N-acetylglucosamine. The hydrolyzing mechanism involved the reverse of anomeric configuration at the cleavage site. Aglycon is displaced by a water molecule, which requires the presence of an acid to attack the glycosidic bond on one side and a base to activate a water molecule on the other side of the bond.

The tertiary structure of barley seed chitinase (Hart et al., 1995) has been determined (Figure 1.4.).

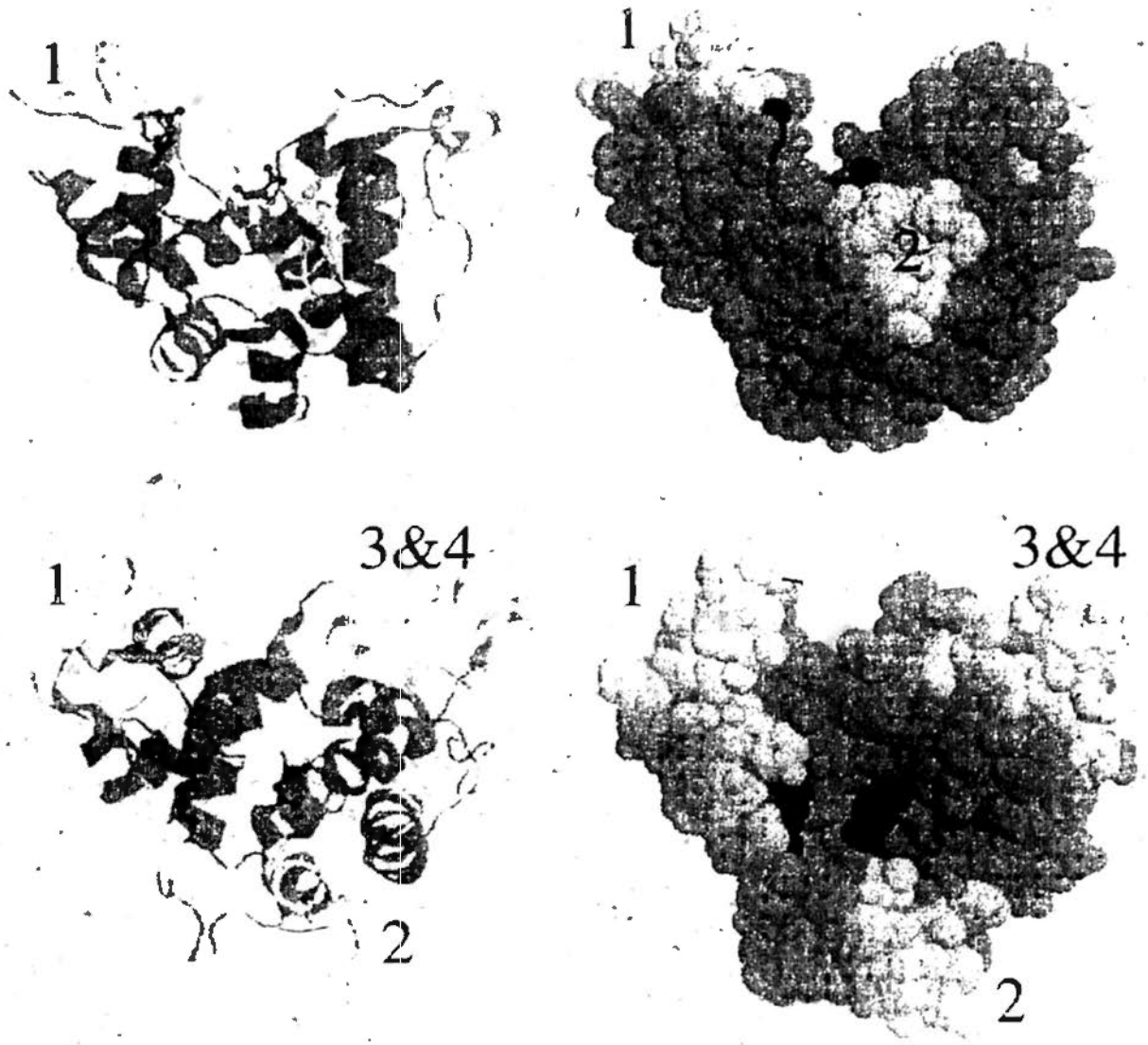


Figure 1.4. Tertiary structure of barley seed chitinase. The ribbons and space-filling model of barley seed chitinase are shown on the left-hand side and right-hand side respectively. The top pictures, which emphasize the cleft, are shown from the front. The bottom pictures, which are shown in a perpendicular view, provide a view of the cleft.

Table 1.3. Example of chitinases.

Source	Reference
Stomach of silver croaker *Pennahia argentatus*	Ikeda et al., 2009
Bacillus thuringiensis	Liu et al., 2009
Tamarind (*Tamarindus indica*) seeds	Patil et al., 2009
Aeromonas schubertii	Liu et al., 2009
Bacillus licheniformis SK-1	Kudan and Pichyangkura, 2009
Penicillium sp. LYG 0704	Lee et al., 2009
Red algae, *Chondrus verrucosus*	Shirota et al., 2008
Tex6 Maize (*Zea mays*) kernels	Moore et al., 2004

1.2.1.2. Biological Properties of Chitinases

The growth of fungi is strongly suppressed by chitinases, as chitin is a major structural polysaccharide in the cell wall. The chitinase caused thinning of the growth tip of the fungus, *Trichoderma longibrachiatum*, which swelled and then the hyphae bursted. The growth of the fungus was inhibited eventually (Arloria et al., 1992). Nevertheless, not all chitinases have antifungal activity, e.g. class IIb chitinases. Transgenic plants have been produced to express high levels of endochitinase CHIT42 were resistant to the soil pathogen *Rhizoctonia solani* (Kern et al., 2009). The lipo-oligosaccharide nodulation factors were recognized as modified chitin oligomers mediating recognition and morphogenesis, which disclosed chitinases and chitin-binding proteins as receptors or modulators of the nodulation in legumes (Denarie et al., 1993). A class IV chitinase was identified as a differentiation factor in embryogensis in the carrot (De Jong et al., 1992). A chitinase from the chive *Allium tuberosum* displayed antiproliferative effect on breast cancer cells and HIV-1 reverse transcriptase inhibitory activity (Lam et al., 2000). Chitinases are anti-freeze proteins in winter rye (Hon et al., 1995). Some chitinases are aboundant in seeds or vegetative tissues and may work as storage proteins (Rao and Gowda, 2008).

1.2.2.1. Overview of Ribonucleases (RNases)

Ribonucleases are enzymes that can cleave the phosphodiester bond of nucleic acids. RNases were purified from plants, fungi and animals (Table 1.4). Ribonucleases can be classified into RNase T1 family and RNase T2 family, according to the base specificity and molecular weight of ribonucleases.

RNase T1 is a guanylic acid-specific RNase with a molecular weight around 11 kDa. RNase T1 family can be divided into two subfamilies, RNase T1 subfamily and barnase subfamily. The RNase T1 subfamily can be further divided into five groups according to the location of the disulfide bond. The orthogonal views showing the structural elements of barnase is displayed in Figure 1.5. (Kraulis, 1991).

The action mechanism of RNase is an acid-base catalyst that involves Glu-58 and one of the two histidine residues, His-40 and His-92. Firstly, Glu-58 performs as a base and removes the proton from the 2' –OH of guanylic acid, and the proton on His-40 or His-92, acts as an acid catalyst, is transferred to the leaving nucleotides. Secondly, His-40 or His-92 performs as a base and activates a water molecule, and then Glu-58 performs as proton donor (Takahashi and Moore, 1982).

Aspergillus oryzaer RNase is an adenylic acid-specific RNase with a molecular weight around 36 kDa (Uchida and Egami, 1971), which was classified as RNase T2. At that time, RNase T2-like enzymes were defined as acidic RNases without base

specificity, and with a molecular weight over 24 kDa. They were isolated from various bacteria, animals, plants and viruses. There are two identical sequences with 9 and 12 amino acid residues found in RNases T2 family.

The mechanism is an acid-base catalysis, which involves His-46 and His-109. His-109 removes the hydrogen from the 2'-OH of the ribose moiety, which attack the positively polarized P atom while His-46 performs as a hydrogen donor. His-104 is the anion-binding site of the phosphate group and so polarizes the phosphate moiety. Glu-105 also polarizes the phosphodiester bond and stabilizes the pentacovalent intermediate in the transition state.

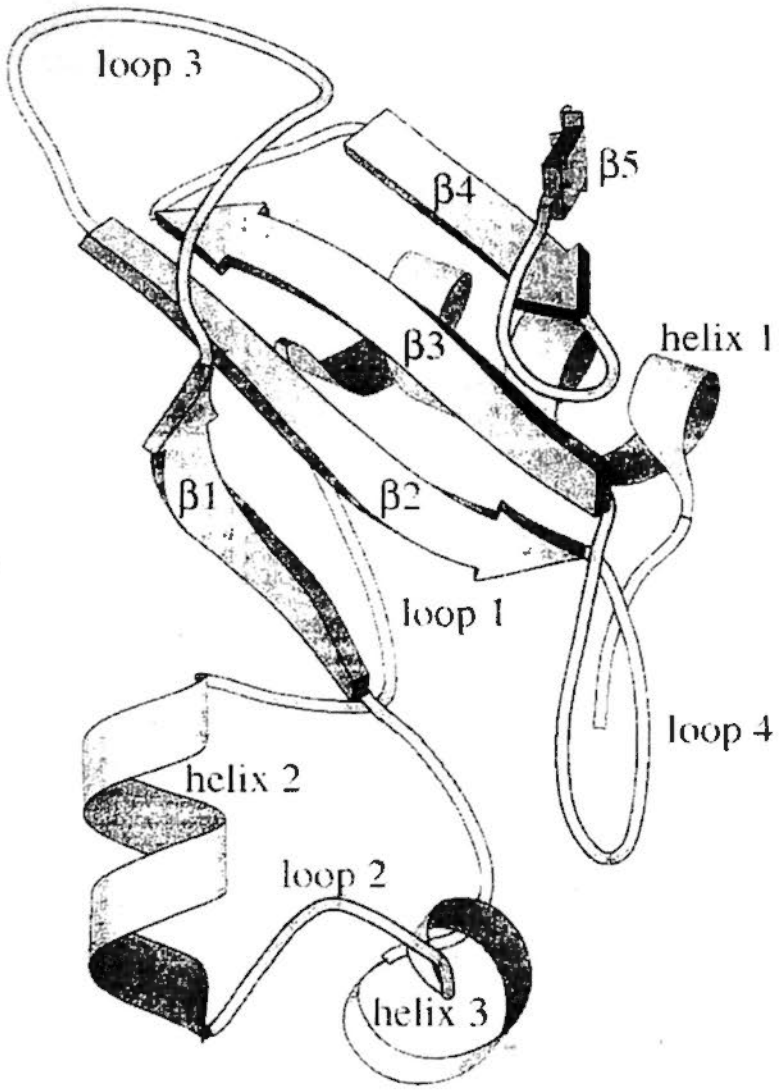


Figure 1.5. Orthogonal views showing the structural elements of barnase.

Table 1.4. Examples of ribonucleases.

Source of ribonuclease	Reference
Red king crab *Paralithodes camtschatica*	Menzorova et al., 2009
Bacillus cereus	Zhou and Niu, 2009
Staphylococcus aureus	Chevalier et al., 2008
Rana pipiens oocytes	Ardelt et al., 2008
Astragalus mongholicus	Yan et al., 2008
Hypsizigus marmoreus	Guan et al., 2007
Green turtle (*Chelonia mydas*) egg white	Katekaew et al., 2006

1.2.2.2. Biological Activities of Plant RNases

RNases can increase the efficiency of phosphate recycling. The secreted fungal RNases can promote the degradation of soil organic matter into phosphate form and so make it ready for absorption (Fraser and Low, 1993). The phosphate in RNA in extracellular space during damage, senescence, or programmed cell death can be degraded and released by extracellular RNases. Moreover, vacuolar RNases may participate in the recycling of intracellular RNA in plant cells vacuoles (Abel et al., 1990).

Cellular structures are dismantled and macromolecules are disassembled to small molecules for relocation to other organs during senescence (Stoddart and Thomas, 1982). During leaf and flower senescence and endosperm mobilization, RNase LX is the degradation enzymes specifically produced (Lehmann et al, 2001).

Programmed cell death is essential during physiological processes and developmental patterns in many organisms (Vaus, 1993), especially during plant development and defense against plant pathogens (Greenberg, 1994; 1996). One of the best examples is differentiation of xylem (Lehmann et al, 2001).

The activities of RNase are raised in infected tissues and wounding tissues (Green, 1994). RNases (Farkas et al., 1982) and other plant defense proteins like β-1,3-glucanases and chitinases (Mauch and Staehelin, 1989) are also assembled.

The storage of a high concentration of these defense proteins in vacuoles can provide a rapid response when pathogens attack.

Onconase, purified from frog embryo RNase, suppressed the proliferation of tumor cell at low concentrations from 0.2-5 μg/ml (Darzynkiewicz et al., 1988) and destroyed the cells at higher concentrations from 10-100 μg/ml (Wu et al., 1993). Onconase could bind to the cell surface, and reach the cytosol where RNA is enriched. Onconase could increase the survival time of mice bearing M109 Madison carcinoma (Mikulski et al., 1990) and patients in phase 1 clinical trials (Mikulski et al., 1993). However, kidney damage, as evidenced by edema and proteinuria, was observed.

1.2.3.1. Overview of Ribosome-Inactivating Proteins (RIPs)

RIPs are N-glycosidases. The adenine residue in the mammalian 28S rRNA of the large ribosomal subunit can be cleaved by RIPs. As a result, protein synthesis is inhibited. RIPs have been isolated from flowering plants (Fong et al., 1991), found in bacteria and fungi. Examples of RIPs are listed in Table 1.5.

There are two kinds of subunits, A chain and B chain, which are N-glycosidases and galactose-specific lectin, respectively. RIPs are classified according to the kind of subunit(s) that they own (Barbieri et al., 1993). There is only an A chain in type I RIPs with a molecular weight ranging from 26 kDa to 30 kDa. Most of RIPs belong to type I. Type II RIPs have both A chain and B chain, linked by disulfide bond. There are two A chains and two B chains in type IV RIPs (Citores et al., 1993). Type III RIPs are synthesized RIPs, one example is maize b-32 RIP, which is synthesized as a proenzyme, activated after the removal of a short internal peptide segment leaving two segments of 16.5 and 8.5 kDa (Walsh et al., 1991).

RIPs have N-glycosidase activity, which is a unique property. Endo and his colleagues disclosed that the 28S rRNA was modified by ricin specifically. Endo's band was observed after treatment with ricin A-chain on rat ribosome and rRNA (Figure 1.6.). Different RIPs have specific deadenylation site. Finally, protein synthesis is inhibited (Endo et al., 1987).

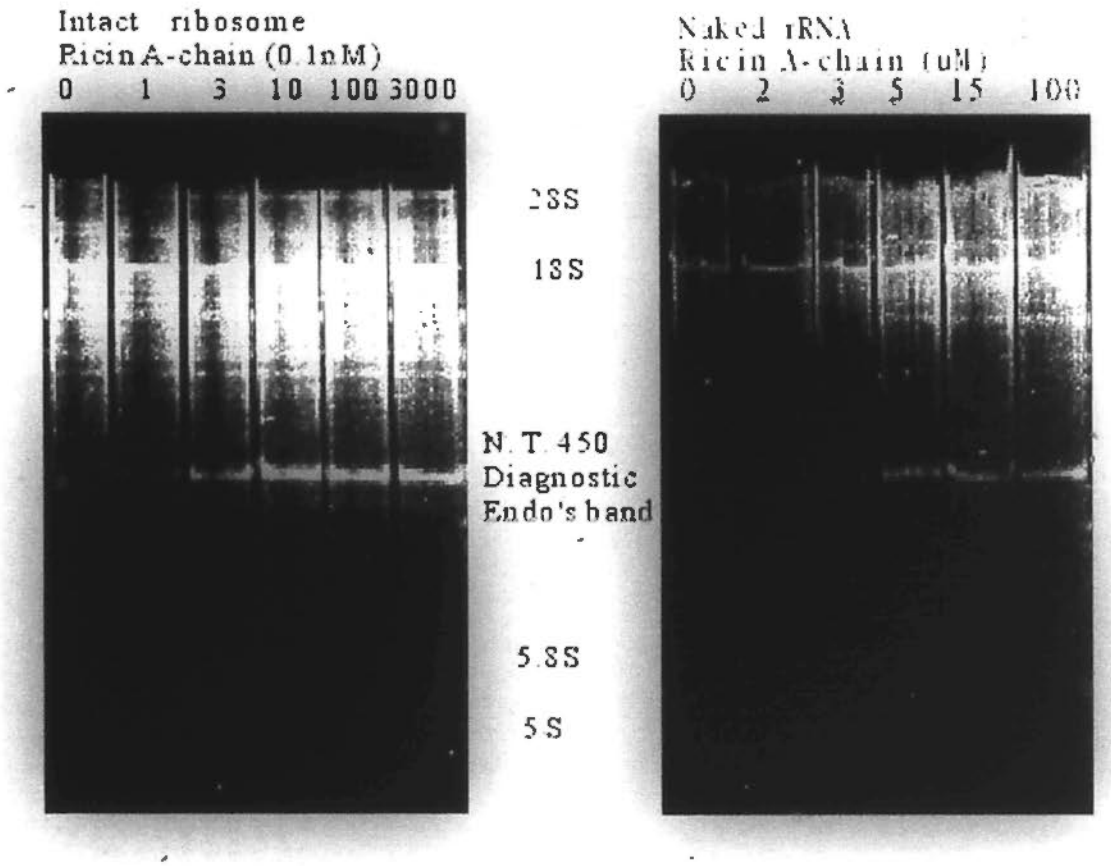


Figure 1.6. Effect of different concentrations of ricin A-chain on rat ribosome and rRNA. The samples were analyzed by electrophoresis in 2.5% polyacrylamide, 0.5% agarose composite gel. The RNA fragment, Endo's band, formed after aniline treatment (Endo et al., 1987).

Trichosanthin could induce the necrosis of the syncytiotrophoblasts of placental villi. As a result, clotting in local circulation was induced and caused a large area of infarction. Then, the level of human chorionic gonadotropin and steroid hormones declined. Finally, the suppression of metabolites exchange induced abortion (Chang et al., 1979).

RIPs can inhibit the early step in the immune response (Rock et al., 1993). It was reported that type I RIPs showed higher toxicity to macrophages than lymphocytes (Spreafico et al., 1983).

RIPs showed anti-viral activity. Selective permeability ability was changed by the virus in virus-infected cells. Therefore, RIPs can enter the virus-infected cells easily, and inhibit the protein synthesis. Finally, the replication of virus was suppressed (Zhao et al., 2010).

Table 1.5. Examples of ribosome-inactivating proteins.

Source of RIP	Reference
Trichosanthes kirilowii Maxim	Shu et al., 2009
Hypsizigus marmoreus fruiting bodies	Wong et al., 2008
Abrus pulchellus seeds	Castilho et al., 2008
Cucurbitaceae	Zhang and Halaweish, 2007
Momordica cochinchinensis seeds	Chuethong et al., 2007
Phytolacca heterotepala leaves	Di Maro et al., 2007
Lychnis chalcedonica seeds	Chambery et al., 2006
Amaranthus tricolor leaves	Roy et al., 2006
Charybdis maritima agg	Touloupakis et al., 2006
Ximenia americana	Voss et al., 2006
Cucurbita moschata	Barbieri et al., 2006

1.2.3.2. Roles of RIPs in Plants

When the virus was inoculated into the leaves of *Phytolacca americana*, which contains the RIP, Pokeweed antiviral protien (PAP), the degree of viral infection declined (Stevens et al., 1981). Cereal RIPs have antifungal activity (Roberts and Selitrennikoff 1986), which can inhibit the growth of fungal pathogens (Leah et al., 1991).

RIPs are stored in the subcellular compartments, which act as a suicidal agent in cells. When a pathogen or virus infects the cell, the RIPs are released. The ribosomes are then inactivated, viral protein synthesis is suppressed and viral replication is suppressed (Taylor et al., 1993).

RIPs are activated and enter the cytoplasm when the leaves of both *Phytolacca americana* and *Hura crepitans* were undergoing senescence or programmed cell death, being infected or under stress (Stirpe et al., 1996).

1.2.3.3. Possible applications of RIPs

Protein synthesis of virus-infected cells can be inhibited by RIPs by impairing the ribosomes, but normal cells are not affected. The antiviral property of RIPs empowers them to become antiviral agents, e.g. *Bougainvillea xbuttiana* antiviral RIP (Choudhary et al., 2008).

Immunotoxins are chimeric molecules. They are synthesized by linking the

protein toxin with a monoclonal antibody or cell-binding ligand. So they can specifically remove undesired cells, e.g. ricin A chain (Lamb et al., 1985). But cytokines released, endothelial damage and other side effects would be induced. And they can prevent and cure graft-versus-host diseases (Ghetie et al., 1994).

Several immunotoxins can specifically and effectively destroy acutely and persistently HIV-infected cells by directing different regions of the HIV envelope glycoprotein (gp41 and gp120) and surface antigens (CD4 and CD25) with immunotoxin (Van Oijen and Preijers 1998).

The antiviral and anti-fungal activities of RIPs enable the possibility of application in agriculture. A gene coding for an antifungal RIP from *Phytolacca heterotepala* leave was expressed in tobacco to increase resistance against different fungal pathogens (Corrado et al., 2005), to increase the crop yield markedly.

1.2.4.1. Overview of the PR (Pathgenesis-Related)-5 Family: Thaumatin-Like Proteins (TLPs)

The N-terminal sequences of PR-5 proteins are similar to thaumatin, as a result, they are called thaumatin-like proteins (TLPs). They were isolated from different parts of plants, including fruits (Pressey, 1997), seeds (Chu and Ng, 2003) and leaves (Hon et al., 1995) (Table 1.6). TLPs normally cannot be found in healthy plants, but the concentration of TLPs increase rapidly under biotic or abiotic stress. In the greenhouse-grown tomato, the concentration of TLP, osmotin, in roots is higher than those in leaves and stems, is possibly due to the exposure to soil-associated microbial (Rodrigo et al., 1991).

The 3-dimensional structure of one Pinus monticola TLP is shown in Figure 1.7. (Liu et al., 2010).

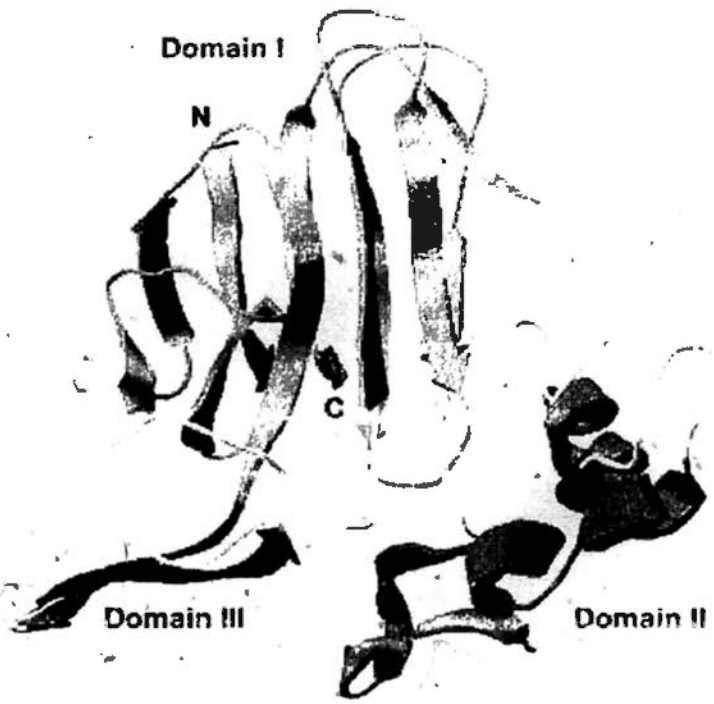


Figure 1.7. Three-dimensional (3D) structure of one Pinus monticola TLP. There are 11 β-sheets and a β-sandwich in domain I, 3 α-helical structures plus 2 β-sheets in domain II and a junction loop and a β-sheet in domain III (Liu et al., 2010).

1.2.4.2. Biological Properties of TLPs

Tomato TLP, osmotin, and grape TLP displayed antifungal activities toward *Phomopsis viticola* and *Botrytis cinerea* mycelia. Moreover, they can suppress spore germination and germ tube growth of *U. necator*, *Phomopsis viticola*, and *Botrytis cinerea*. Therefore, they can protect grapevine from fungal attack (Monteiro et al., 2003).

The cold-acclimated winter rye (*Secale cereale* L.) leaves anti-freeze protein showed a N-terminal sequence highly similar to barley and rice TLPs. It can control formation of extracellular ice during freezing. It is essential to the survival of freezing-tolerant plants (Hon et al., 1995).

1.2.4.3. Biotechnological Application – Transgenic Plants

Zeamatin, osmotin and other thaumatin-like proteins have been shown to have antifungal activity against different kinds of fungi. The genomic clones for thaumatin-like proteins empowered the expression of TLPs at high levels in transgenic plants.

Rice is one of the most important crops in the world. The basic food of more than two billion people is rice. However, because of biotic and abiotic stresses, the yield of rice is significantly decreased. The most destructive biotic stress is sheath blight disease caused by *Rhizoctonia solani*. Antifungal rice chitinase and thaumatin-like protein were transgenic in elite indica rice cultivars in order to overcome sheath blight disease (Maruthasalam et al., 2007).

Table 1.6. Examples of thaumatin-like proteins (TLPs)

Source of TLP	Reference
Grape juice	Van Sluyter et al., 2009
Latex of *Carica papaya*	Looze et al., 2009
Jelly fig (*Ficus awkeotsang*) Achenes	Chua et al., 2007
Emperor banana fruits	Ho and Ng, 2007
Cassia didymobotrya cell culture	Vitali et al., 2006
Kweilin chestnut (*Castanopsis chinensis*) seeds	Chu and Ng, 2003
Kiwi fruit	Wang and Ng, 2002
Ripe banana fruit	Barre et al., 2000

1.2.5.1. Overview of Protease Inhibitors

Protease inhibitors can inhibit the proteolytic activity of proteases. The mode of inhibition can be competitive or non-competitive. The first trypsin inhibitor was characterized in 1946 (Kunitz, 1946). Protease inhibitors have been isolated from plants (Torres-Castillo et al., 2009), animals (Khan and Bano, 2009) and microorganisms (Gesner-Apter and Carmeli, 2009).

According to the mechanism of protease inhibitors, they can be divided into serine protease inhibitors, cysteine protease inhibitors, aspartic protease inhibitors and metalloprotease inhibitors (Table 1.7).

Protease inhibitors can be classified into Kunitz type trypsin inhibitors, Bowman-Birk protease inhibitors and squash inhibitors according to their molecular masses, number of disulfide bridges and protease specificity. The classification of protease inhibitors is summarized in Table 1.8.

Table 1.7. Examples of different kinds of protease inhibitors.

	Source	Reference
Serine protease inhibitor	*Monacrosporium cystosporium*	Yang et al., 2008
	King cobra venom	He et al., 2008
	Radianthus macrodactylus	Sokotun et al., 2007
Cysteine protease inhibitor	Ostrich skeletal muscle	Tshidino et al., 2009
	Spodoptera litura	Arora et al., 2009
	Coconut endosperm	Panicker et al., 2009
Aspartic protease inhibitor	Latex of *Ficus racemosa* (L.)	Devaraj et al., 2008
	Aspergillus fumigatus	Vickers et al., 2007
	Bacillus licheniformis	Kumar and Rao, 2006
Metalloprotease inhibitor	*Photorhabdus luminescens*	Valens et al., 2002
	Erinaceus europaeus	Omori-Satoh et al., 2000
	Galleria mellonella	Wedde et al., 1998

Table 1.8. Classification of protease inhibitors into Kunitz type trypsin inhibitors, Bowman-Birk protease inhibitors and squash inhibitors

	Molecular mass	Number of disulfide bridge	Specificity	Source
Kunitz Type Trypsin Inhibitor	~ 20 kDa	2	Trypsin	*Pithecellobium dumosum* seeds (Oliveira et al., 2009)
Bowman-Birk Protease Inhibitor	~ 8 kDa	7	Trypsin, chymotrypsin	*Lupinus albus* seeds (Scarafoni et al., 2008)
Squash Inhibitor	~ 3 kDa	-	Trypsin, plasmin, blood blotting factors	*Echinocystis lobata* seeds (Stachowiak et al., 1996)

1.2.5.2. Possible Applications of Protease Inhibitors

Soybean cysteine protease inhibitor and pepstatin A (soybean Kunitz trypsin inhibitor) were fed to cowpea bruchids. Soybean protease inhibitor and pepstatin A, can inhibit the growth of cowpea bruchids. A synergistic effect was observed when a combination of protease inhibitors was fed (Amirhusin et al., 2007).

Some of the protease inhibitors display anti-microbial activity. Cysteine protease inhibitor from pearl millet exhibits potent antifungal activity against *Trichoderma reesei* etc (Joshi et al., 1998). Potide-G isolated from potato tubers suppressed the proteolytic activity of trypsin, chymotrypsin and papain. Furthermore, it potently suppressed growth of a variety of bacterial (e.g. *Staphylococcus aureus*) and fungal (e.g. *Rhizoctonia solani*) strains (Kim et al., 2006a). The expression of rice cysteine proteinase inhibitor gene could protect transgenic tobacco plants from tobacco etch virus and potato virus Y (Gutierrez-Campos et al., 1999).

Phytocystatins (cysteine proteinase inhibitors) are involved in the regulation of protein turnover and defense against pathogens and insects. It can inhibit the stored cysteine proteases so as to control the rate of germination and growth of seedlings (Hwang et al., 2009).

The proliferation of over 60 cancer cell lines has been reported to be inhibited by protease inhibitors. Clinical testing of nelfinavir has been performed in cancer

patients (Bernstein and Dennis, 2008). Populations consuming a diet rich in legumes, which are known to have a high content of trypsin inhibitors, have a reduced incidence of malignant disease (Losso, 2008). Furthermore, telaprevir (Gentile et al., 2009) and boceprevir (Mederacke et al., 2009) are used for the treatment of hepatitis C virus infection.

Different combinations of protease inhibitors are used for treatment of HIV: lopinavir/ritonavir (Barragan and Podzamczer, 2008), lopinavir/ritonavir or ritonavir-boosted atazanavir (Elliott and Pujari, 2008). Boosted saquinavir and atazanavir are generally safe for mothers during pregnancy and children (van der Lugt et al., 2008). However, drug resistance to HIV-1 protease inhibitors has been found (Nalam and Schiffer, 2008). Toxicity-associated adverse events remain a major concern when prescribing HIV protease inhibitors (Boesecke and Cooper, 2008). So, the identification of more protease inhibitors should continue.

Protease inhibitors have been proposed as potential defense molecules for increased insect resistance in crop plants. The over-expression of the potato PI-II and carboxypeptidase inhibitors results in increased resistance to *Heliothis obsoleta* and *Liriomyza trifolii* larvae in transgenic tomato (Abdeen et al., 2005). Combined expression of defense genes with different action mechanisms may provide a better protection to the plants.

1.2.6. Unclassified Antifungal Proteins

In order to classify a plant defense protein, the complete amino acid sequence (or at least N-terminal sequence) should be obtained and compared with other known classified plant defense proteins by "Protein BLAST" search. Most of the plant defense proteins fall into the groups of lectins, hemagglutinins, chitinases, ribonucleases, ribosome-inactivating proteins, thaumatin-like proteins, protease inhibitors, proteases, defensins, thionins, cyclophilin-like proteins, killer protein, hemolysin, etc.

However, there are still many proteins with defensive properties, e.g. antifungal, anti-viral or anti-bacterial activities, which displayed N-terminal sequences not falling into any groups of plant defense proteins, they are generally called antifungal proteins.

Novel antifungal proteins have been purified from different sources, e.g. pumpkin rinds (Park et al., 2009). A list of novel antifungal proteins is summarized in Table 1.10. The discovery and investigation of unclassified antifungal proteins can widen the potential application of antifungal proteins.

Table 1.10. Examples of unclassified antifungal proteins.

Source	N-terminal Sequence (Sequence-related protein)	Fungal Species Inhibited	Reference
Pumpkin rinds	QGIGVGDNDGKRGKR	*Botrytis cinerea, Colletotrichum coccodes, Fusarium solani, Fusarium oxysporum, Trichoderma harzianum*	Park et al., 2009
Brassica alboglabra seeds	PEGPFQGPKATKPGDLAXQTWGGWX	*Fusarium oxysporum, Helminthosporium maydis, Mycosphaerella arachidicola, Valsa mali*	Lin et al., 2008
Passion fruit seeds	PSERCRRQMQGDFS (2S albumin)	*Trichoderma harzianum, Fusarium oxysporum, Aspergillus fumigatus*	Pelegrini et al., 2006
Kiwi fruits	GAKRAYDEVEAQN	*Fusarium oxysporum*	Xia and Ng, 2004
Polyporus alveolaris fruiting bodies	GVCDMADLA	*Botrytis cinerea, Fusarium oxysporum, Mycosphaerella arachidicola, Physalospora piricola*	Wang et al., 2004
Pleurotus eryngii fruiting bodies	ATRVVYCNRRSGSVVFFDDTVYYEG	*Fusarium oxysporum, Mycosphaerella arachidicola*	Wang and Ng, 2004
Black pumpkin seeds	PQRGEGGRAGNLLREEQEI	*Botrytis cinerea, Fusarium oxysporum and Mycosphaerella arachidicola*	Wang and Ng, 2003

Table 1.10. Examples of unclassified antifungal proteins (continued).

Source	N-terminal Sequence (Sequence-related protein)	Fungal Species Inhibited	Reference
Green chickpea seeds	VKSTGRADDDLAVKTKYLPP	*Botrytis cinerea, Mycosphaerella arachidicola, Physalospora piricola*	Chu et al., 2003
Passion fruit seeds	QSERFEQQMQGQDFSHDERFLSQAA (2S albumin)	*Fusarium oxysporum, colletotrichum lindemuthianum*	Agizzio et al., 2003
Chickpea seeds	GVGYKVVVTTTAAADDDDVV ARCENFADSYRQPPISSSQQT	*Mycosphaerella arachidicola, Fusarium oxysporum, Botrytis cinerea*	Ye et al., 2002
Pinto bean seeds	GARKDDHAKLVFLLKDIEYQ	*Fusarium oxysporum, Physalospora piricola*	Ye and Ng, 2002
Ceylon spinach seeds	GADFQECMKEHSQKQHQHQG (*Arabidopsis thaliana* DNA-binding protein)	*Botrytis cinerea, Mycosphaerella arachidicola, Fusarium oxysporum*	Wang and Ng, 2001a
Peanut	KSPYYQKKTENPQAQRQLQSDDQEP (Peanut allergen)	*Mycosphaerella arachidicola, Fusarium oxysporum, Coprinus comatus*	Ye and Ng, 2001

1.2.7. Aims of Study

All living organisms develop their defense systems. One of the mechanisms developed in plants is producing plant defense protein(s). They can be grouped into different classes. Interestingly, most of them shared the same kinds of properties, for example, anti-viral activity and antifungal activity. Some of them may have other biological activities, e.g. anti-tumor activity. The discovery of new plant defense proteins and understanding of action mechanism made the application of such proteins feasible.

In this study, two lectins were purified from *Capparis spinosa* seeds and *Hibiscus mutabilis* seeds. A hemagglutinin was isolated from *Phaselous vulgaris*, cultivar "French bean 35". A novel dimeric β-lactoglobulin-like antifungal protein and an antifungal amidase were purified from *Passiflora edilus* seeds and *Peltophorum pterocarpum* seeds, respectively. The biological activities of the above proteins were investigated and discussed.

Chapter 2: Isolation and Characterization of a Lectin with Potentially Exploitable Activities from Caper (*Capparis spinosa*) Seeds

2.1. Introduction

Capparis spinosa is a plant belonging to Family *Capparaceae*. Various parts of this plant have been shown to have biological activity. Its bud extract inhibits the replication of *Herpes simplex* virus type 2 and upregulates the expression of pro-inflammatory cytokines including interleukin-12, interferon-γ, and tumor necrosis factor-α (Arena et al., 2008). The principal antioxidant in the buds have been identified to be flavanols and hydroxycinnamic acid (Bonina et al., 2002). p-Methoxybenzoic acid has been identified as an antihepatotoxic component in the methanol-soluble fraction of the aqueous extract (Gadgoli and Mishra, 1999). The aqueous extract demonstrated hypolipidemic (Eddouks et al., 2005) and anti-hyperglycemic (Eddouks et al., 2004) activities. Other activities comprised antiviral, immunomodulatory (Arena et al., 2008), chondrocyte protective (Panico et al., 2005), anti-allergeric, anthihistaminic (Trombetta et al., 2005), antifungal (Ali-Shtayeh and Abu Ghdeib, 1999), anti-Leishmania (Jacobson and Schlein, 1999), and antimicrobial (Mahasneh, 2002) activities, and also inhibitory effect on fibroblast proliferation and type 1 collagen production in progressive systemic sclerosis (Cao et al., 2008).

An indication of the possible presence of a lectin in *C. spinosa* is found in the demonstration of the ability of specific carbohydrates to inhibit the effect of *C. spinosa* in agglutinating and killing parasites (Jacobson and Schlein, 1999). However, the lectin has not been purified. In view of the dearth of information on proteinaceous constituents of *C. spinosa*, the present investigation was undertaken to isolate *C. spinosa* lectin and compare its characteristics with known lectins.

2.2. Materials and Methods

2.2.1. Purification of Lectin

Fresh seeds (580 g) were collected from caper (*Capparis spinosa).* They were extracted by homogenizing in distilled water. Following centrifugation at 20,000 g for 30 minutes at 4 °C, Tris-HCl buffer (2 M, pH 7.4) was added to the supernatant until the final concentration of Tris reached 20 mM. The supernatant was then applied on a 5 cm x 9 cm column of DEAE-cellulose (Sigma). Unadsorbed proteins were eluted with 20 mM Tris-HCl buffer (pH 7.4). Adsorbed proteins were eluted stepwise, first with 0.1 M NaCl and then with 1 M NaCl added to the 20 mM Tris-HCl buffer. The fraction eluted with 1 M NaCl was taken, dialyzed, and then subjected to chromatography on a 5 cm x 4.5 cm column of SP-Sepharose (GE Healthcare). After removal of unadsorbed proteins with 20 mM Tris-HCl buffer (pH 7.4), adsorbed proteins were eluted with 1 M NaCl added to the 20 mM Tris-HCl

buffer. The adsorbed fraction was saved and dialyzed extensively against water and lyophilized before chromatography on a 0.34 ml CIM-QA (Convective Interaction Media-Quaternary Amine) column (BIA Separations) using an AKTA Purifier (GE Healthcare). Unadsorbed proteins were removed with 20 mM Tris-HCl (pH 7.4) buffer. Adsorbed proteins were eluted, first with a linear 0 - 0.3 M NaCl gradient, and then with 1 M NaCl in the 20 mM Tris-HCl (pH 7.4) buffer. The fraction eluted at 0.15 - 0.25 M NaCl was saved, dialyzed and lyophilized before re-dissolving and chromatography on a Superdex 75 10/300 GL column using an AKTA Purifier (GE Healthcare). The column had been calibrated with molecular mass markers, including Blue Dextran 2000 (to indicate void volume), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), myoglobulin (17.6 kDa), ribonuclease A (13.7 kDa), aprotinin (6.5 kDa) and vitamin B12 (1.3 kDa) (GE Healthcare), was conducted to determine the molecular mass of the protein. The first fraction collected represented purified lectin.

2.2.2. Molecular mass determination using sodium dodecyl sulfate–polyacrylamide gel electrophoresis, gel filtration, and N-terminal amino acid sequencing.

The purified lectin was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli and Farve, 1973). Gel filtration on a fast protein liquid chromatography Superdex 75 10/300 GL column, was conducted to determine the molecular mass of the lectin. The N-terminal sequence of the lectin was determined as described in the established mehod (Wong and Ng, 2006).

2.2.3. Protein determination

Protein concentration was determined by Bradford reagent (dye-binding method) using bovine serum albumin as standard.

2.2.4. Assay for hemagglutinating activity

In the assay for hemagglutinating activity, a serial twofold dilution of the sample solution in microtiter U-plates (50 µl) was mixed with 50 µl of a 2 % suspension of rabbit red blood cells in phosphate-buffered saline (pH 7.2) at room temperature. The results were read after about 1 hour when the blank had fully sedimented. The hemagglutination titer, defined as the reciprocal of the highest dilution exhibiting hemagglutination, was reckoned as one hemagglutination unit. Specific activity is the number of hemagglutination units per milligram protein (Wong and Ng, 2006).

2.2.5. Inhibition of hemagglutination by carbohydrates

The inhibition of hemagglutination was carried out as follows (Wong and Ng, 2006). The carbohydrates tested included D-glucosamine, mannitol, D-xylose, sucrose, D-fucose, D-raffinose, α-lactose, D-fructose, L-arabinose, galactonic acid, D-galacturonic acid, D-galactose, D-mannose, D-glucuronic acid, D-glucose and D-sorbitol. A serial twofold dilution of the carbohydrate solution in microtiter U-plates (25 µl) was mixed with 25 µl sample solution (8 hemagglutination units) and incubated for 30 min. A 50 µl 2% suspension of rabbit erythrocytes was added. After further 30 min room temperature incubation, the concentrations of carbohydrate(s) that can inhibit the agglutination can be determined.

2.2.6. Effects of temperature and pH on lectin-induced hemagglutination

The sample (50 µg/ml) was incubated at various temperatures (4, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 °C) or buffers of various pH values (pH 1 - pH 14) for 15 minutes. It was then cooled down to room temperature or neutralized to pH 7 respectively immediately before hemagglutination assay.

2.2.7. Assay of antifungal activity

The assay for antifungal activity was executed with agar diffusion assay using

100 × 15 mm petri plates containing 10 ml of potato dextrose agar. After the mycelial colony had developed, sterile blank paper disks (0.625 cm in diameter) were placed around and at a distance of 1 cm away from the rim of the mycelial colony. An aliquot of tested sample or nystatin (as positive control) in 20 mM PBS (pH 6.0) was introduced to a disk. The plates were incubated at room temperature until mycelial growth had enveloped peripheral disks containing the control (buffer) and had produced crescents of inhibition around disks containing samples with antifungal activity. The phytopathogenic fungi tested including *Mycosphaerella arachidicola*, *Fusarium oxysporum*, *Helminthosporium maydis*, *Valsa mali* and *Rhizoctonia solani* (Wang and Ng, 2006). The fungi were provided by Department of Microbiology, China Agricultural University, China. Q-Sepharose, Phenyl-Sepharose, DEAE-cellulose, and Superdex 75 10/300 GL were from GE Healthcare, Hong Kong.

The IC_{50} value for the antifungal activity of the sample was done as described in (Wang and Ng, 2006). Different concentrations of protein were added separately to aliquots, each containing 2 ml potato dextrose agar at 45 °C, mixed quickly, and decanted into separate 3.3-cm petri dishes. After the agar had cooled down, a small amount of mycelia was inoculated. Buffer only served as a negative control. The dishes were incubated at room temperature. The area of the mycelial colony was measured and the IC_{50} value for the antifungal activity of acafusin against the fungus

was determined. IC_{50} is the concentration of antifungal protein required to produce 50 % reduction of the area of mycelial colony.

2.2.8. Assay of HIV-1 reverse transcriptase inhibitory activity

The assay of the protein for the ability to inhibit HIV-1 reverse transcriptase was examined using an enzyme-linked immunosorbent assay kit from Boehringer Mannheim (Germany) (Wong and Ng, 2006). The assay takes advantage of the ability of reverse transcriptase to synthesize DNA, begining from the template/primer hybrid poly(a)·oligo (dT) 15. Digoxigenin- and biotin-labeled nucleotides in an optimized ratio are incorporated into one and the same DNA molecule freshly synthesized by the reverse transcriptase (RT). To detect and quantify synthesized DNA as a parameter for RT activity a sandwich enzyme-linked immunosorbent assay protocol was followed. Biotin-labeled DNA binds to the surface of microtiter plate modules that have been precoated with streptavidin. Next, an antibody to digoxigenin, conjugated to peroxidase (anti-DIG-POD), binds to the digoxigenin-labeled DNA. Finally, the peroxidase substrate is added. The peroxidase enzyme catalyzes the cleavage of the substrate, forming a colored reaction product. The absorbance of the samples at 405 nm can be determined using a microtiter plate (ELISA) reader and is directly proportional to the level of RT activity. A fixed quantity (4-6 ng) of

recombinant HIV-1 reverse transcriptase was employed. The inhibitory activity of the protein was calculated as percent inhibition in comparison with a control without the protein. *Brassica campestris* lipid transfer protein (Lin et al., 2007) was used as positive control.

2.2.9. Assay of antiproliferative activity

Breast cancer MCF-7 cells (ATCC) and HepG2 cells (ATCC) were suspended in RPMI medium and adjusted to a cell density of 100,000 cells/ml. A 100 µl aliquot of this cell suspension was seeded in a well of a 96-well plate, followed by incubation at 37 °C for 4 hours. Different concentrations of sample or doxorubicin (as positive control) in 100 µl RPMI medium were then added to the wells and incubated for 48 hours. After removing the medium, 30 µl of 5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma) in PBS were spiked into each well. After further incubation for 4 hours, dimethyl sulfoxide (150 µl) was added to each well to dissolve the MTT-formazan at the bottom of the well. After 10 min, absorbance at 595 nm was measured by using a microplate reader (Wong and Ng, 2006).

2.2.10. Annexin-V and propidium iodide (PI) staining

Phosphatidyl serine externalization (loss of membrane asymmetry) was studied by using the annexin-V-FITC and PI staining method. Cells (5×10^5) were plated in a 6-well culture plate and incubated with different concentrations of protein for 24 hours. Then the cells were trypsinized before centrifugation at 2000 g for 4 min. The cells were rinsed with 1 ml PBS and then spun down at 2000 g for 4 min. Cells were resuspended in 250 µl binding buffer (10 mM HEPES, pH 7.4; 140 mM NaCl; 25 mM $CaCl_2$) and then stained with 250 µl staining solution [247 µl binding buffer containing 2.5 µl Annexin V solution (BD Phamingen, CA, USA) and 0.5 µl PI (Sigma)] in darkness for 20 min at room temperature. The sample was analyzed with FACSortTM flow cytometer (Becton Dickinson, Cowley, UK). The signal was detected by FL-1 (530 nm) channel and data were analyzed with the program WinMDI (Version 2.8, Joseph Trotter, La Jolla, CA, USA) (Lai et al., 2006).

2.3. Results

Ion exchange chromatography of the seed extract on DEAE-cellulose produced a very large unadsorbed fraction (D1) and two adsorbed fractions (D2 eluted with 0.1 M NaCl and D3 eluted with 1 M NaCl) of approximately the same size. Hemagglutinating activity resided only in fraction D3 (Figure 2.1). This fraction was separated on SP-Sepharose into a large unadsorbed fraction (SP1) devoid of hemagglutinating activity and a smaller adsorbed fraction (SP2) with hemagglutinating activity (Figure 2.2). Fraction SP2 was subsequently resolved on CIM-QA into a small unadsorbed fraction (Q1) and several adsorbed fractions (Q2 – Q5). Hemagglutinating activity was confined to an adsorbed fraction Q4 eluted toward the second half of the 0 – 0.3 M NaCl gradient (Figure 2.3). This active fraction was subjected to final purification on Superdex 75 10/300 GL. Two fractions, S1 and S2, were obtained. Hemaggulatinating activity resided in the first fraction (S1), which appeared to be much smaller than the second fraction (S2) (Figure 2.4). Fraction S1 was re-chromatographed on Superdex 75 10/300 GL. It was eluted as a single homogeneous peak with a molecular weight of 62 kDa. It demonstrated a single 31-kDa band in SDS-PAGE (Figure 2.5). A summary of the purification of the lectin is included in Table 2.1. The lectin was obtained with a specific activity of 35000 titer/mg and a 100-fold purification. The N-terminal sequence of the lectin

was homologous to other *Phaseolus* lectins or hemagglutinins (Table 2.2). Raffinose, α-lactose, rhamnose and D(+)galactose were the most potent in inhibiting hemagglutination induced by the isolated lectin and inhibition was discernible at 1 mM concentration. L(+)arabinose and D(+)glucosamine were inhibitory at 25 mM and 100 mM concentrations, respectively. Other sugars tested were inactive at 100 mM. The hemagglutinating activity of the lectin was stable throughout the pH range 1 – 12 and completely destroyed at pH 13 and 14. It was stable in the temperature range 0 – 40 °C. The hemagglutinating activity was reduced to half at 50 °C and demolished at 60 °C. The lectin inhibited proliferation of HepG2 and MCF-7 tumor cells with an IC_{50} near 2 μM. Phosphatidyl serine externalization was observed in treated MCF-7 tumor cells (Figure 2.6). The percentage of apoptotic cells was increased by 34 % when the cells were incubated with 30 μM lectin for 24 hours. The lectin inhibited HIV-1 reverse transcriptase with an IC_{50} of 0.28 μM. It inhibited mycelial growth in *V. mali* with an IC_{50} of 18 μM (Figure 2.7) but not in *M. arachidicola, F. oxysporum, H. maydis,* and *R. solani* (not shown).

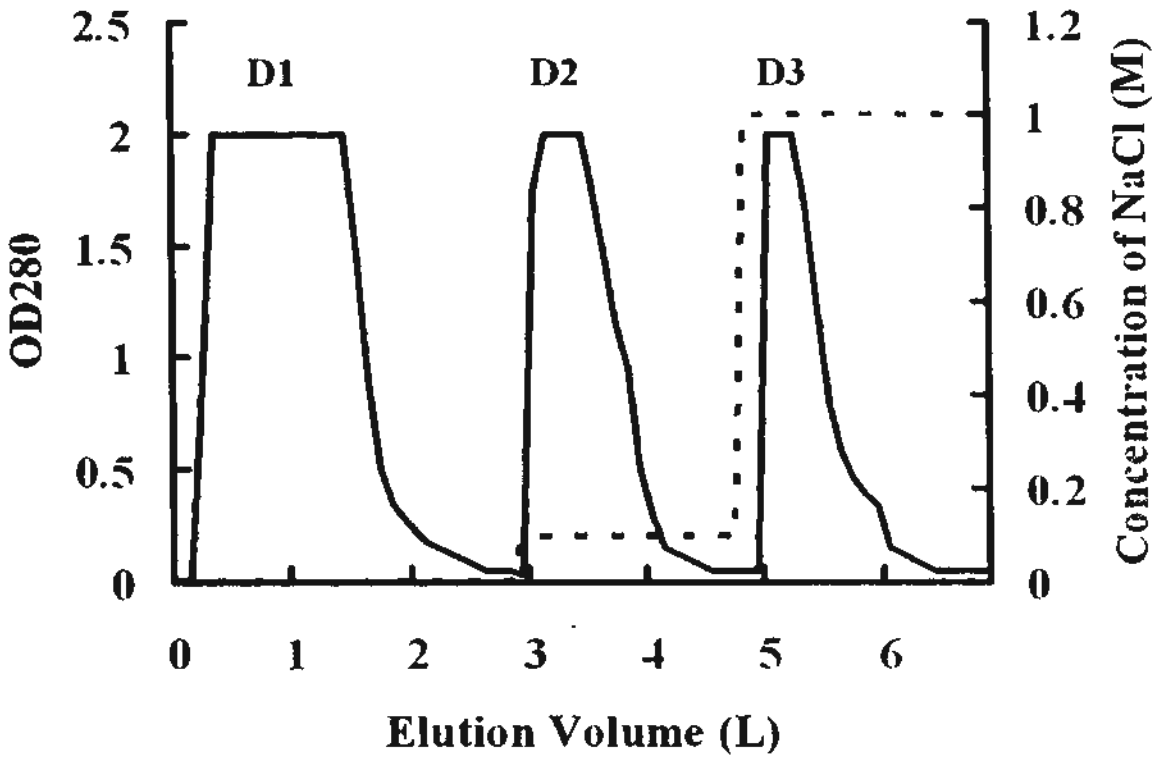


Figure 2.1. Ion exchange chromatography of *Capparis spinosa* seed crude extract on DEAE-cellulose (5 cm x 9 cm). The dotted line indicates the use of 0.1 M NaCl and 1 M NaCl to elute fractions D2 and D3, respectively. Flow rate = 10 ml/min.

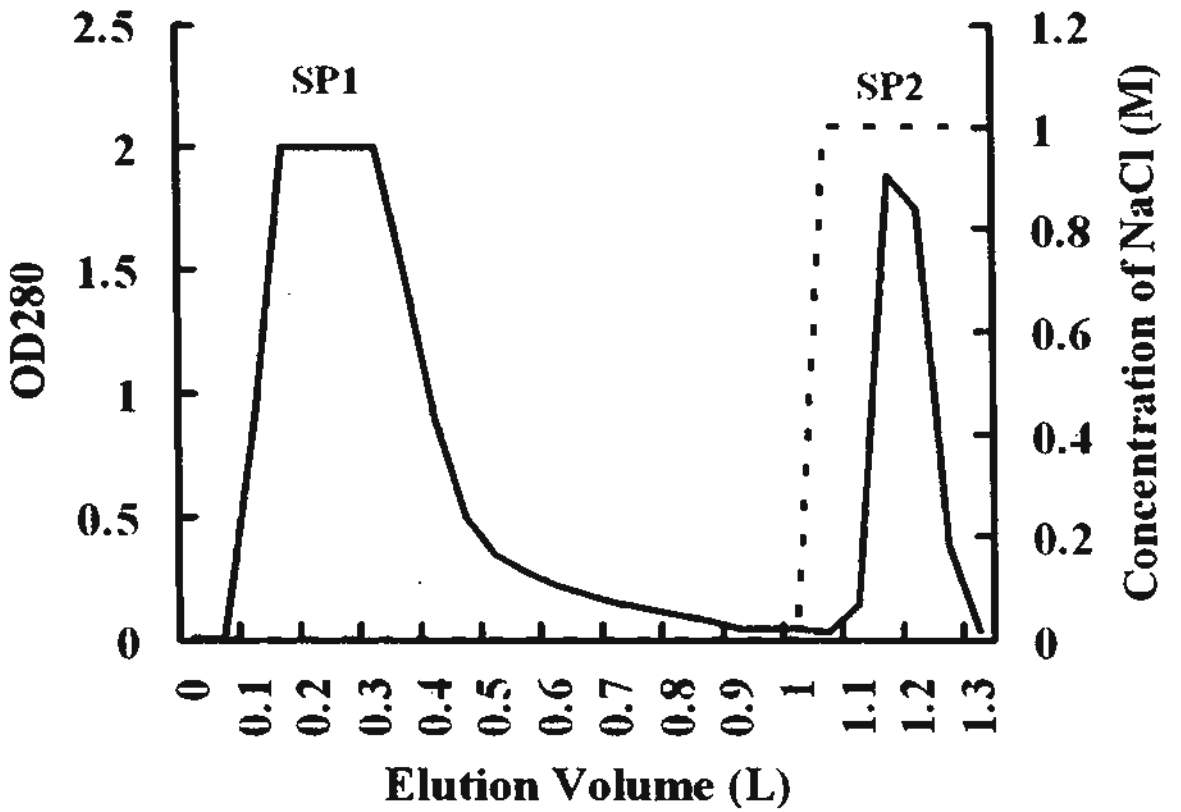


Figure 2.2. Ion exchange chromatography of D3 fraction on SP-Sepharose (5 cm x 4.5 cm). The dotted line indicates the use of 1 M NaCl to elute fractions SP2. Flow rate = 10 ml/min.

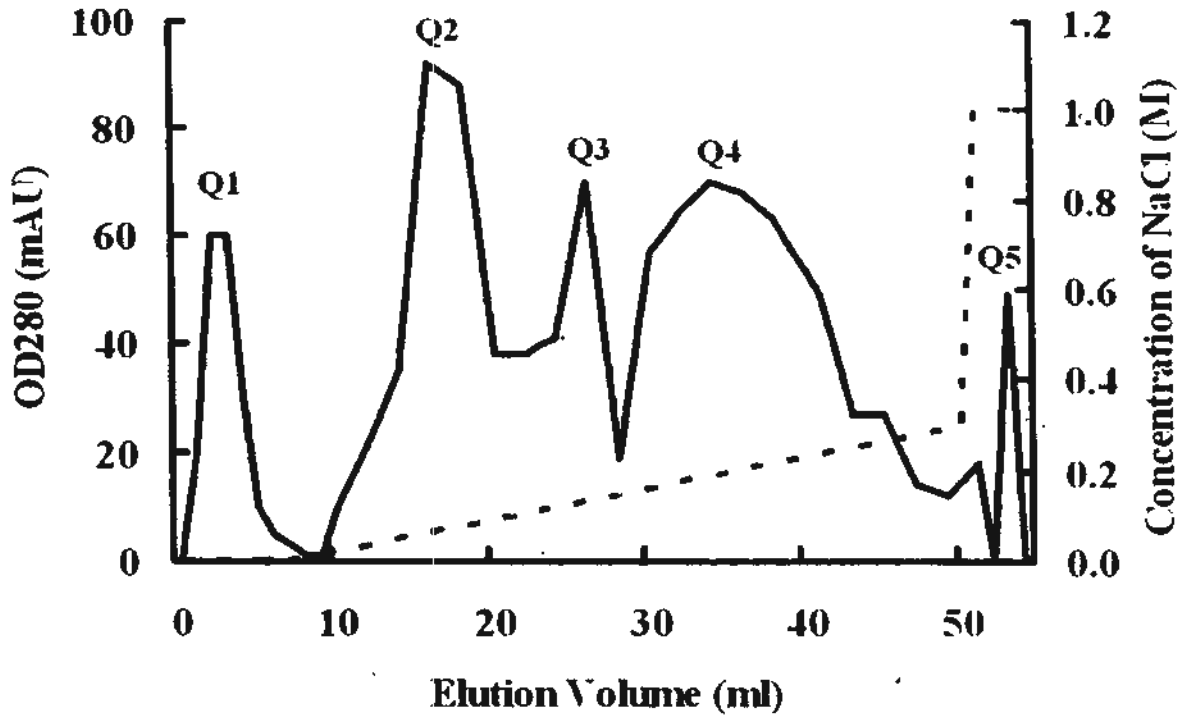


Figure 2.3. Ion exchange chromatography of fraction SP2 on CIM-QA (0.34 ml). The dotted line indicates the use of a linear 0 – 0.3M NaCl gradient to elute fractions Q2 to Q4, and the use of 1 M NaCl to elute fraction Q5. Flow rate = 5 ml/min.

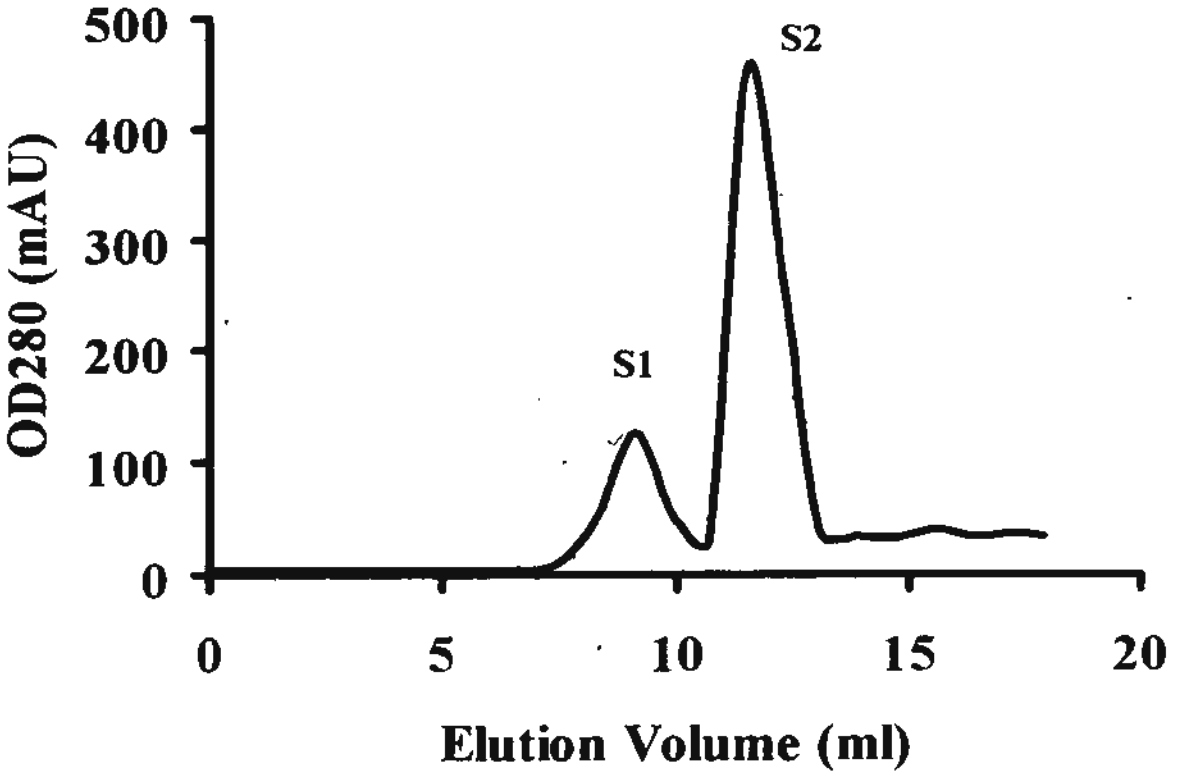


Figure 2.4. Gel filtration of fraction Q4 on Superdex 75 10/300 GL. The first peak represented purified lectins. Flow rate = 0.5 ml/min.

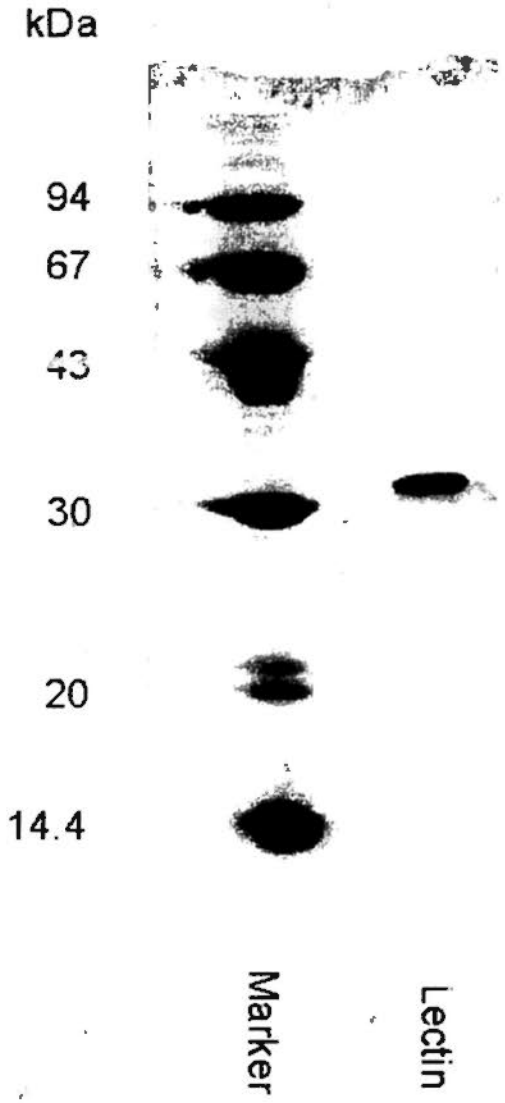


Figure 2.5. SDS-PAGE of purified *Capparis spinosa* lectin. Left lane: Molecular mass markers from GE Healthcare including phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14.4 kDa). Right lane: Fraction S1 representing purified *C. spinosa* lectin.

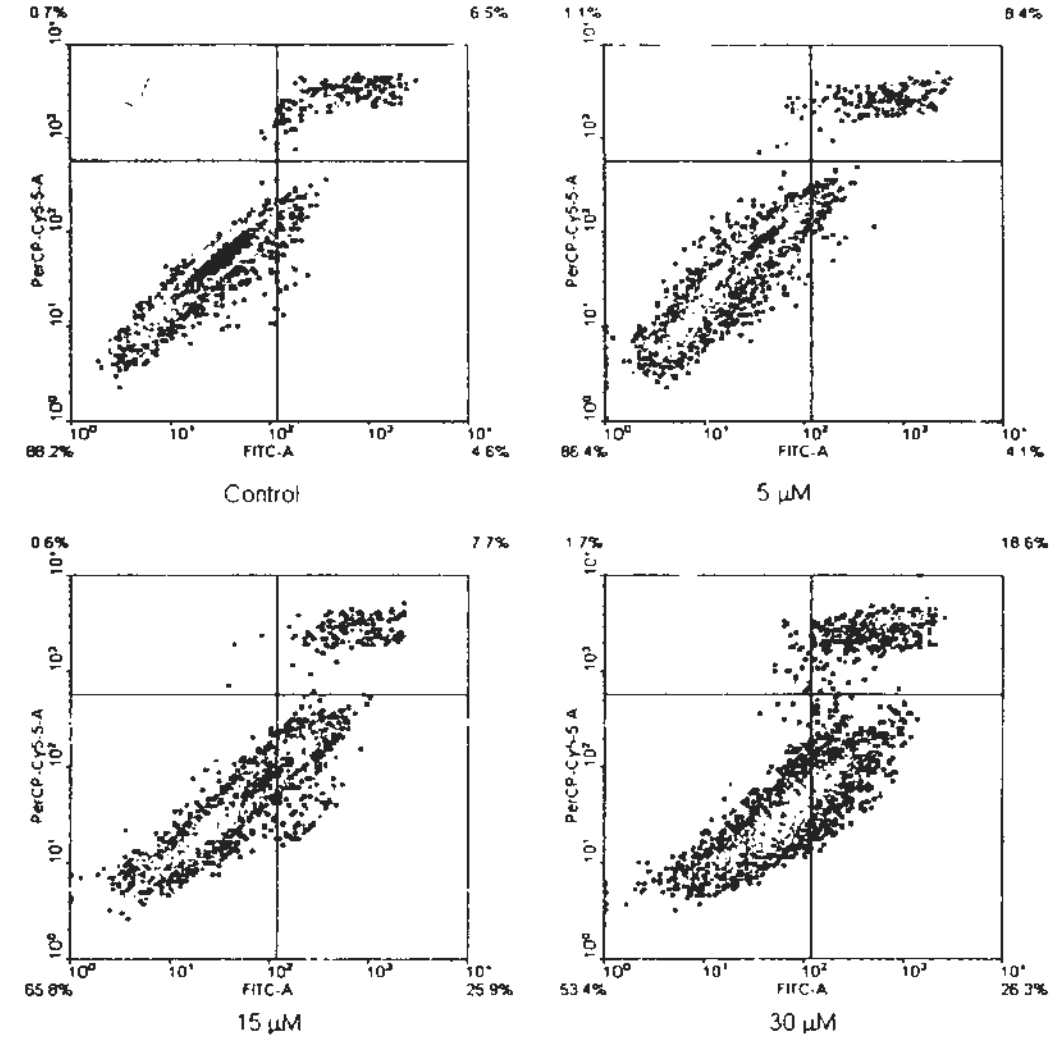


Figure 2.6. Phosphatidyl serine externalization of MCF-7 cells by *Capparis spinosa* lectin. MCF-7 cells were incubated with lectin on a 6-well culture plate for 24 hours. After washing the lectin-treated/ untreated MCF-7 cells with PBS, they were stained with annexin V/PI and then analyzed by flow cytometry. The lower left quadrant shows healthy cells. The upper and lower right quadrants of each plot show annexin V/-PI double-positive cells (i.e. cells undergoing late apoptosis) and annexin V single-positive cells (i.e. cells undergoing early apoptosis), respectively. Data are expressed as means of triplicate. The level of phosphatidyl serine externalization was increased by 34 % when the cells were incubated with 30 μM lectin for 24 hours.

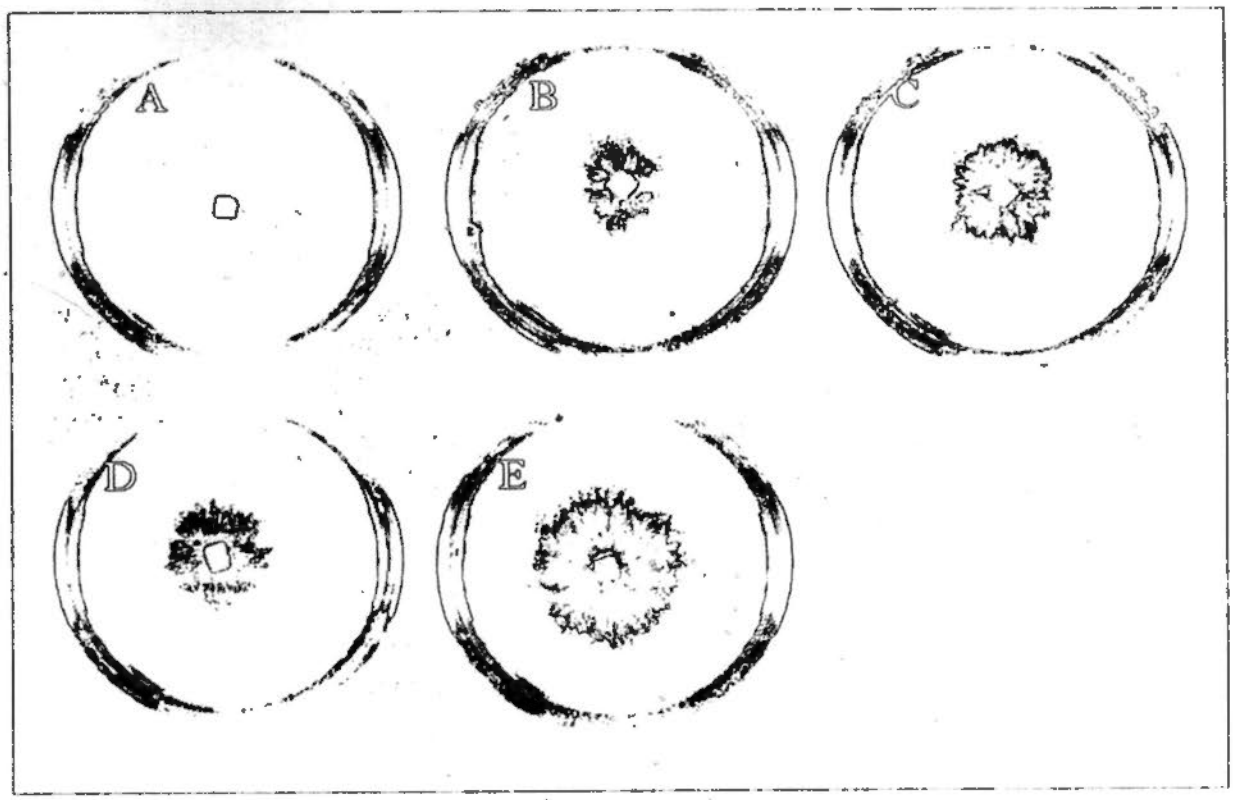


Figure 2.7. Antifungal activity of *Capparis spinosa* lectin toward *Valsa mali*.

A: 100 μM lectin, B: 33 μM lectin. C: 11 μM lectin. D: 3.7 μM lectin. E: buffer control.

Table 2.1. Protein yields from 580 g fresh *Capparis spinosa* seeds and specific hemagglutinating activities (ha) at different stages of purification of *Capparis spinosa* lectin

Column	Fraction collected	Yield (mg)	Specific ha (titer/mg)	Total ha (titer)	Recovery of ha (%)	Purification fold of ha
-	Crude Extract	3000	350	1050000	100	1
DEAE-cellulose	D3	830	1000	830000	79	3
SP-Sepharose	SP2	330	2400	792000	76	7
CIM-QA	Q4	80	9000	720000	69	26
Superdex 75	S1	20	35000	700000	66	100

Table 2.2. N-terminal amino acid sequence of *Capparis spinosa* lectin.

	Residue No.	Sequence
Capparis spinosa Lectin	1	ATETYSGFDA
Phaseolus coccineus lectin	22	ATETSFSFDR
French bean 35 hemagglutinin	1	ATETYSAFQR

Identical amino acid residues are underscored.

2.4. Discussion

Ion exchange chromatography and gel filtration were used to purify *C. spinosa* lectin. The first lectin isolated from Family *Capparaceae*, with a high purification fold (100–fold) compared with 10–fold in the case of French bean lectin (Leung et al., 2008). The subunit molecular mass (31 kDa) and dimeric nature of *C. spinosa* lectin were similar to those of many plant lectins (Ye et al., 2001b). Its N-terminal sequence was similar with other *Phaseolus* lectins or hemagglutinins.

It is of great interest that *C. spinosa* lectin can be inhibited by as many as 6 sugars, including galactose, lactose, arabinose, rhamnose, raffinose, and D(+)glucosamine. It indicated that the *C. spinosa* lectin is a superlectin. French bean lectin cannot be inhibited by simple sugars (Leung et al., 2008). Chive lectin can be inhibited by mannose only (Escribano et al., 2000). Emperor banana lectin can be inhibited by mannose and glucose (Wong and Ng, 2006). *Canavalia gladiata* lectin can be inhibited by mannose, glucose and rhamnose (Wong and Ng, 2005b). *Pleurotus citrinopileatus* lectin can be inhibited by o/p-nitrophenyl-β-d-glucuronide, O/P-nitrophenyl-beta-d- galactopyranoside and maltose, and a polysaccharide, inulin (Li et al., 2008). *C. spinosa* lectin was marked by pronounced pH stability from pH 1 – 12. The lectin had only moderate thermostability up to 40 °C. These characteristics were similar to those of most of the lectins (Leung et al., 2008).

To date only several lectins have been reported with antifungal activity. For example, red kidney bean lectin inhibits *F. oxysporum, Coprinus comatus, and R. solani* (Ye et al., 2001b). The spectrum of antifungal activity of *C. spinosa* lectin (against *V. mali*, but not others) is also distinct from other antifungal lectins. It exhibited potent antiproliferative activity against hepatoma and breast cancer cells, in keeping with similar demonstrations for other lectins (Wong and Ng, 2006). It is noteworthy that it can induce apoptosis in MCF-7 tumor cells. Only galectin-9 (Yamauchi et al., 2006) and mistletoe lectin (Pae et al., 2000) have been shown to induce apoptosis in MCF-7 cells, while there are more reports showing that lectins can induce apoptosis in HepG2 cells, e.g. *Pouteria torta* lectin (Boleti et al., 2008). *C. spinosa* lectin potently inhibited HIV-1 reverse transcriptase with an IC_{50} of 0.28 μM. This activity has been shown by only some lectins with an IC_{50} of 1 - 35 μM.

The distinctive features of *C. spinosa* lectin isolated in this study include (1) a novel N-terminal sequence, (2) pH-stability of hemagglutinating activity, (3) inhibition of hemagglutinating activity by as many as 6 sugars, (4), a diversity of biological activities than other lectins comprising antifungal activity, highly potent HIV-1 reverse transcriptase activity and antiproliferative activity due to induction of apoptosis.

Chapter 3: Novel Galactonic Acid-Binding Hexameric Lectin from *Hibiscus mutabilis* Seeds with Antiproliferative and Potent HIV-1 Reverse Transcriptase Inhibitory Activities

3.1. Introduction

Hibiscus mutabilis belongs to the family Malvaceae. It is commonly known as Confederate rose or cotton rosemallow. Only few publications about *H. mutabilis* were found in a Pubmed search. An aqueous extract of *H. mutabilis* exerted anti-herpes simplex virus-II action (Zheng et al., 1989). *H. mutabilis* is one of the greening tree species planted in a polluted factory area in Shanghai (Yang et al., 2004). The benzene extract of its flowers did not markedly affect pregnancy (Kholkute et al., 1977). Pigments are found in *H. mutabilis* flowers (Yeh et al., 1958).

According to the Herbalist's Manual, *H. mutabilis* leaves aid detoxification, reduce swellings and alleviate pain. *H. mutabilis* leaves can be used to treat carbuncles, dermal swelling or inflammation, scalds, conjunctivitis, external and internal trauma and herpes zoster, its flowers to treat continuous menstrual flow and scalds, and its roots to treat skin ulcer. However, there is no therapeutical information about its seeds.

In view of the paucity of information on *H. mutabilis*, especially with regard to

its proteinaceous constituents, the present investigation was undertaken to isolate a lectin from its seeds. The lectin is multimeric and manifests highly potent HIV-1 reverse transcriptase inhibitory activity and unique sugar specificity. The results of the present study constitute an addition to the scanty literature on hexameric lectins which contrasts sharply with the voluminous data on lectins with a lower molecular mass and a smaller number of subunits.

3.2. Materials and Methods

3.2.1. Purification of lectin

Dried seeds of *H. mutabilis* (420 g) were collected and extracted by homogenization in distilled water (5 ml/g seeds). Following centrifugation at 20000 g for 30 min at 4 °C, ammonium acetate buffer (1 M, pH 4.6) was added to the supernatant until the final concentration of ammonium acetate reached 20 mM. The supernatant was then applied on a 5 cm x 15 cm column of SP-Sepharose (GE Healthcare). Unadsorbed proteins were eluted with 20 mM ammonium acetate buffer (pH 4.6). Adsorbed proteins were eluted with 1 M NaCl added to the 20 mM ammonium acetate buffer. The unadsorbed fraction was dialyzed, lyophylized, and then subjected to chromatography in 100 mM ammonium acetate buffer (pH 4.6) on a Superdex 75 10/300 GL column using an AKTA Purifier (GE Healthcare). The column had been calibrated with molecular mass markers, including Blue Dextran 2000 (to determine void volume), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), myoglobulin (17.6 kDa), ribonuclease A (13.7 kDa), aprotinin (6.5 kDa), and vitamin B12 (1.3 kDa) (GE Healthcare). The first fraction was collected, lyophilized, and then subjected to chromatography in 100 mM ammonium acetate buffer (pH 4.6) on a Superdex 200 HR 10/300 GL column using an AKTA Purifier. The column had been calibrated with molecular mass markers,

including Blue Dextran 2000 (to determine void volume), thyroglobulin (669 kDa), ferritin (440 kDa), immunoglobulin G (150 kDa), bovine serum albumin (67 kDa), β-lactoglobulin (35 kDa), ribonuclease A (13.7 kDa), and aprotinin (6.5 kDa) (GE Healthcare). The first fraction represented purified lectin.

3.2.2. Molecular mass determination using sodium dodecyl sulfate–polyacrylamide gel electrophoresis, gel filtration, and N-terminal amino acid sequencing were described in Section 2.2.2.

3.2.3. Protein determination was described in Section 2.2.3.

3.2.4. Assay for hemagglutinating activity was described in Section 2.2.4.

3.2.5. Inhibition of lectin-induced hemagglutination by divalent metal ions

The purified lectin was demetallized by treatment with 10 mM EDTA overnight. It was dialyzed against deionized water extensively before incubation at room temperature for 15 min in the presence of one of the following salts: $ZnSO_4$, $CaCl_2$, $FeSO_4$, $MgCl_2$, $MgSO_4$, $CuCl_2$, and $CuSO_4$, all at 5 mM. Hemagglutinating assay was then performed (Wong and Ng, 2006).

3.2.6. Inhibition of lectin-induced hemagglutination by carbohydrates was described in Section 2.2.5.

3.2.7. Effects of temperature and pH on lectin-induced hemagglutination were described in Section 2.2.6.

3.2.8. Assay of antifungal activity was described in Section 2.2.6.

3.2.9. Assay of HIV-1 reverse transcriptase inhibitory activity was described in Section 2.2.8.

3.2.10. Assay of antiproliferative activity toward tumor cells was described in Section 2.2.9.

3.3. Results

3.3.1. Isolation of Lectin

The *H. mutabilis* seed extract was resolved on SP-Sepharose into two fractions, an unadsorbed fraction SP1 with hemagglutinating activity and an adsorbed fraction SP2 without hemagglutinating activity (Figure 3.1). Fraction SP1 was dialyzed, and lyophilized before separation on Superdex 75 10/300 GL into three major fractions. S75-1, with hemagglutinating activity eluted in the void volume, and two larger fractions, S75-2 and S75-3, without such activity (Figure 3.2). Fraction S75-1 was lyophylized before being separated on Superdex 200 10/300 GL into two major fractions, S200-1, a larger fraction with hemagglutinating activity, and a smaller fraction S200-2 without activity (Figure 3.3). Fraction S200-1, with a native molecular mass of 150 kDa, represented purified lectin as evidenced by a single 24-kDa band in SDS-PAGE, indicating that the lectin was a hexamer (Figure 3.4). The *H. mutabilis* lectin was obtained with a specific hemagglutinating activity of 4000 unit/mg for rabbit blood. There was no hemagglutinating activity for rat blood and human blood. The lectin yield was 17 mg from 420 g seeds. The recovery of hemagglutinating activity was 34 % (Table 3.1). Its N-terminal sequence, ACVAPLDEAACAAAK, resembled none of the lectins reported so far.

3.3.2. Hemagglutinating activity of lectin

The hemagglutinating activity of the lectin could be inhibited by galactonic acid at 25 mM concentration There was no inhibition when other sugars were tested up to 100 mM. The hemagglutinating activity of the lectin was stable in the pH range 4 – 7. About 50% activity was retained at pH 3 and pH 8 – 9. The activity was completely eliminated at pH 1 – 2, and 10 – 14. The activity was stable in the temperature range 0 °C – 50 °C, reduced to half at 60 °C, and destroyed at 70 °C. Of the various salts tested, only manganese chloride and manganese sulfate restored the activity of the lectin.

3.3.3. Other biological activities of isolated lectin

The lectin inhibited HIV-1 reverse transcriptase with an IC_{50} of 0.2 μM (Figure 3.5). There was no inhibitory effect on mycelial growth of *M. arachidicola, F. oxysporum, H. maydis, V. mali*, or *R. solani* at 100 μg lectin in agar diffusion assay. The lectin exhibited weak antiproliferative activity toward tumor cells. There was about 50 % growth inhibition of MCF-7 cells and about 40 % growth inhibition of HepG2 cells when the cells were treated with 100 μM lectin. *Rachycentron canadum* lectin showed no antiproliferative effect. By comparison, the positive control doxorubicin exhibited an antiproliferative activity toward MCF-7 and HepG2 cells with an IC_{50} of 5 μM and 10 μM, respectively.

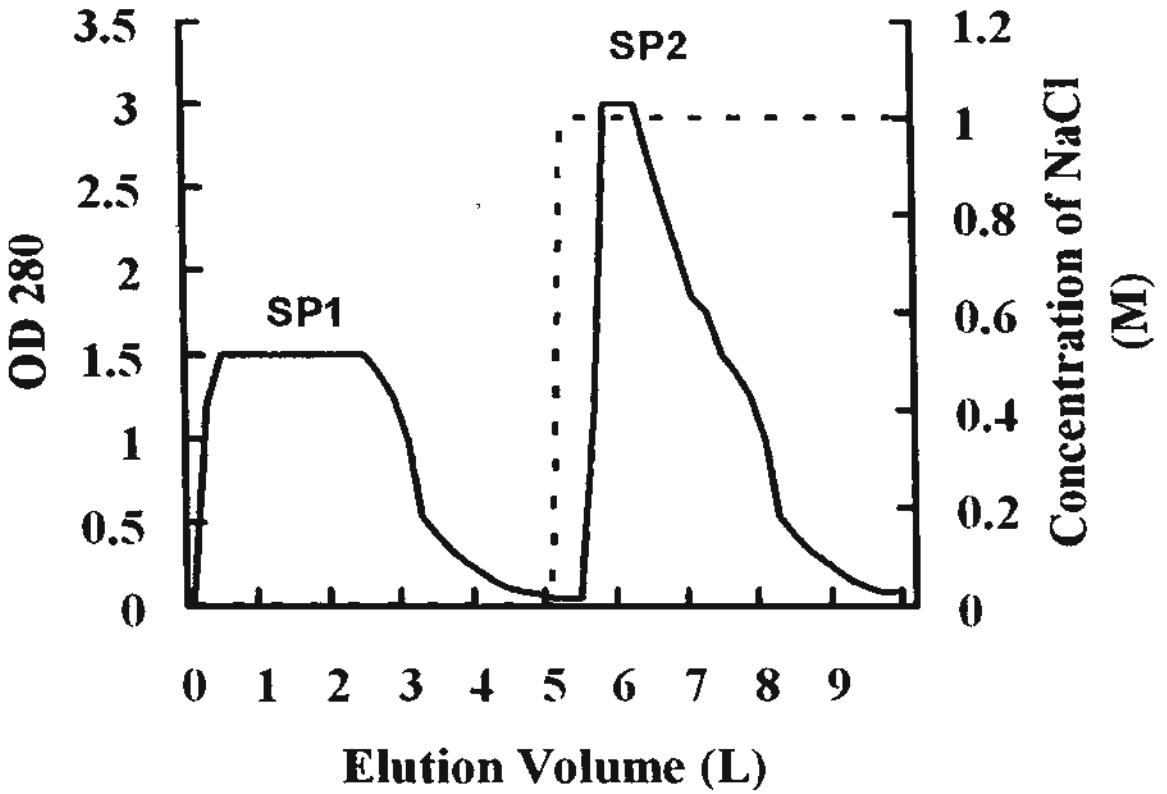


Figure 3.1. Ion exchange chromatography of *Hibiscus mutabilis* extract on SP-Sepharose (5 cm x 15 cm). The dotted line indicates the use of 1 M NaCl to elute fractions SP2. Flow rate = 10 ml/min.

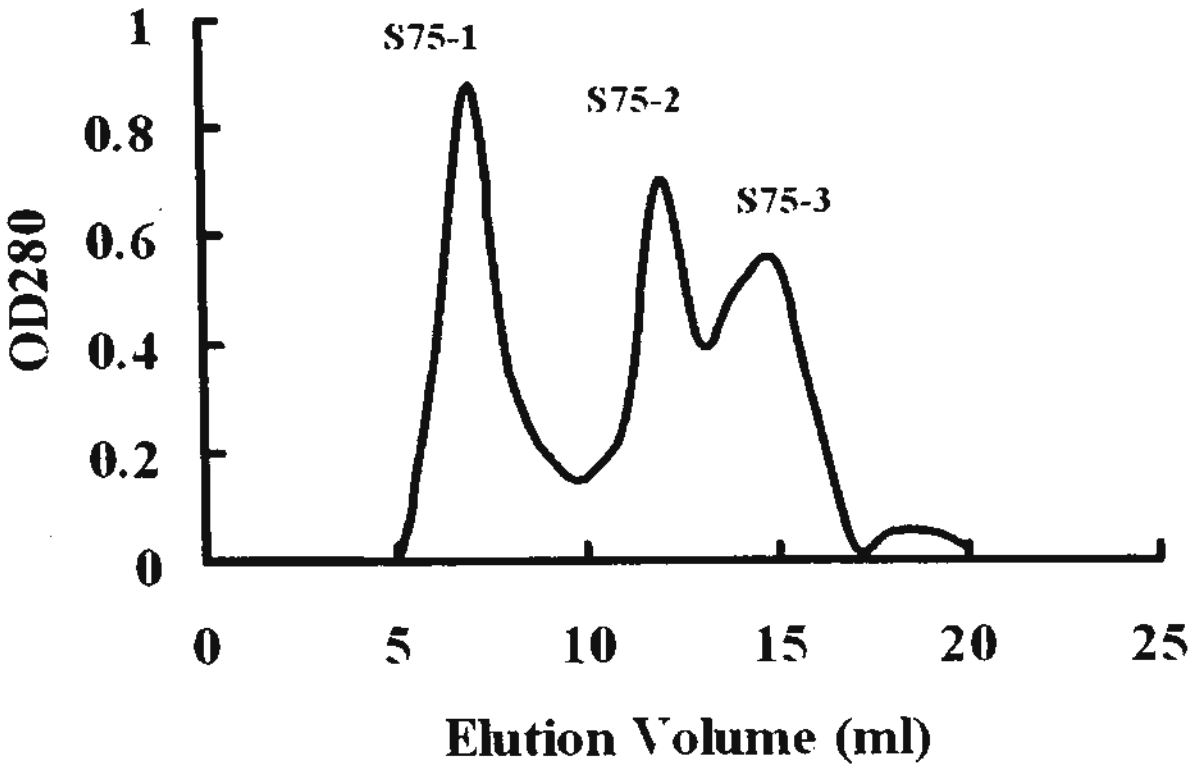


Figure 3.2. Gel filtration of SP1 fraction on Superdex 75 10/300 GL. S75-1, S75-2 and S75-3 were eluted out. The S75-1 is the peak with activity. Flow rate = 0.5 ml/min.

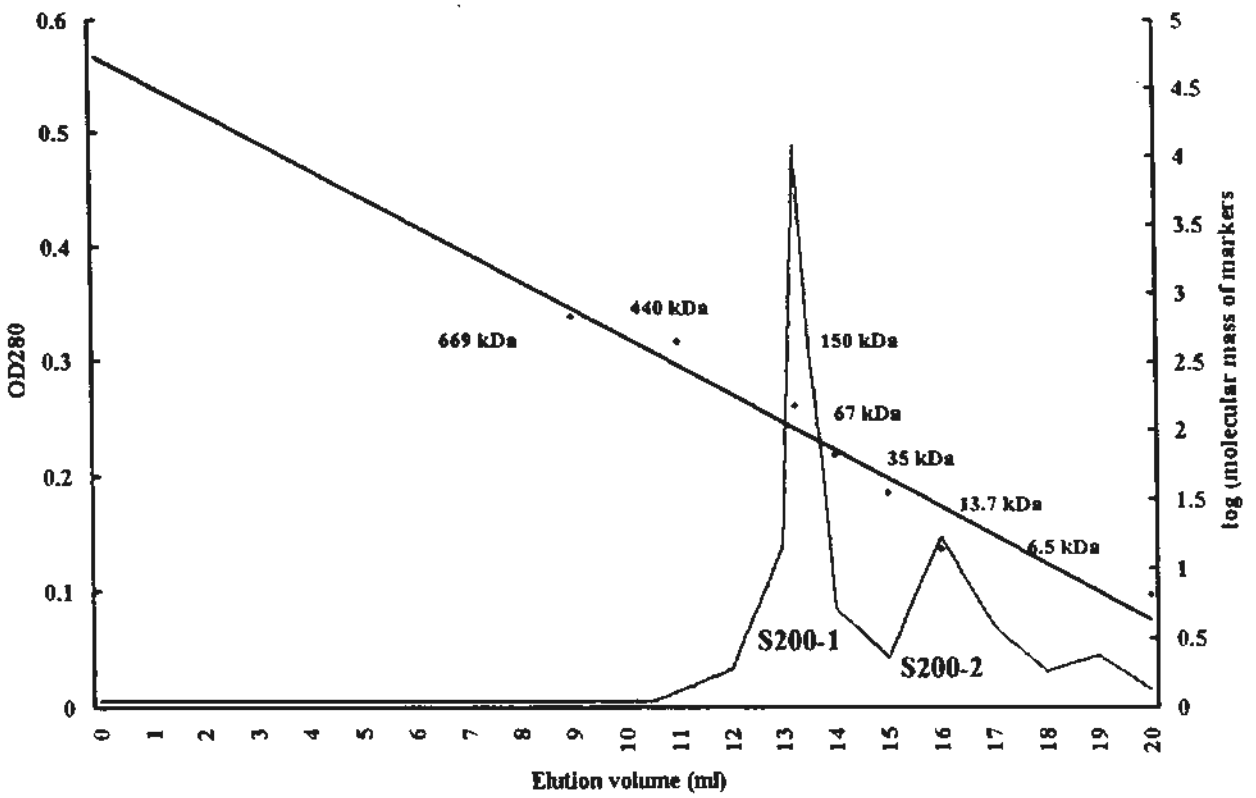


Figure 3.3. Gel filtration of fraction S75-1 on Superdex 200 10/300 GL. S200-1 and S200-2 were eluted out. S200-1 represents purified *Hibiscus mutabilis* lectin. Flow rate = 0.5 ml/min.

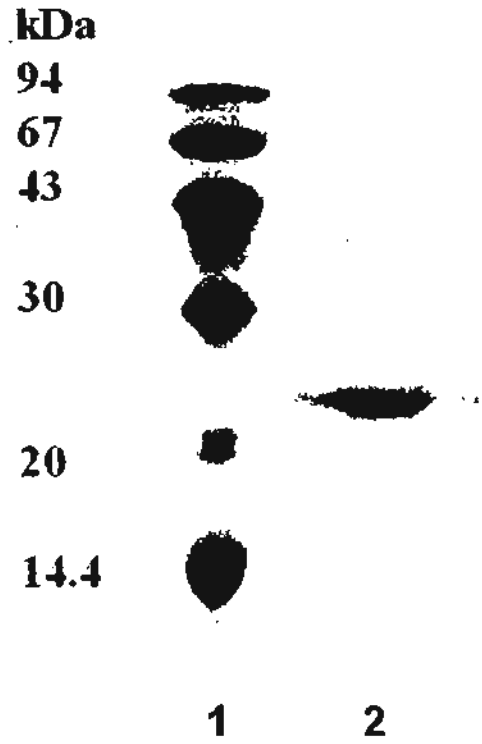


Figure 3.4. SDS-polyacrylamide gel electrophoresis analysis of *Hibiscus mutabilis* lectin.

Lane 1: molecular mass markers from GE Healthcare. Lane 2: Fraction S200-1 (30 μg) representing *H. mutabilis* lectin.

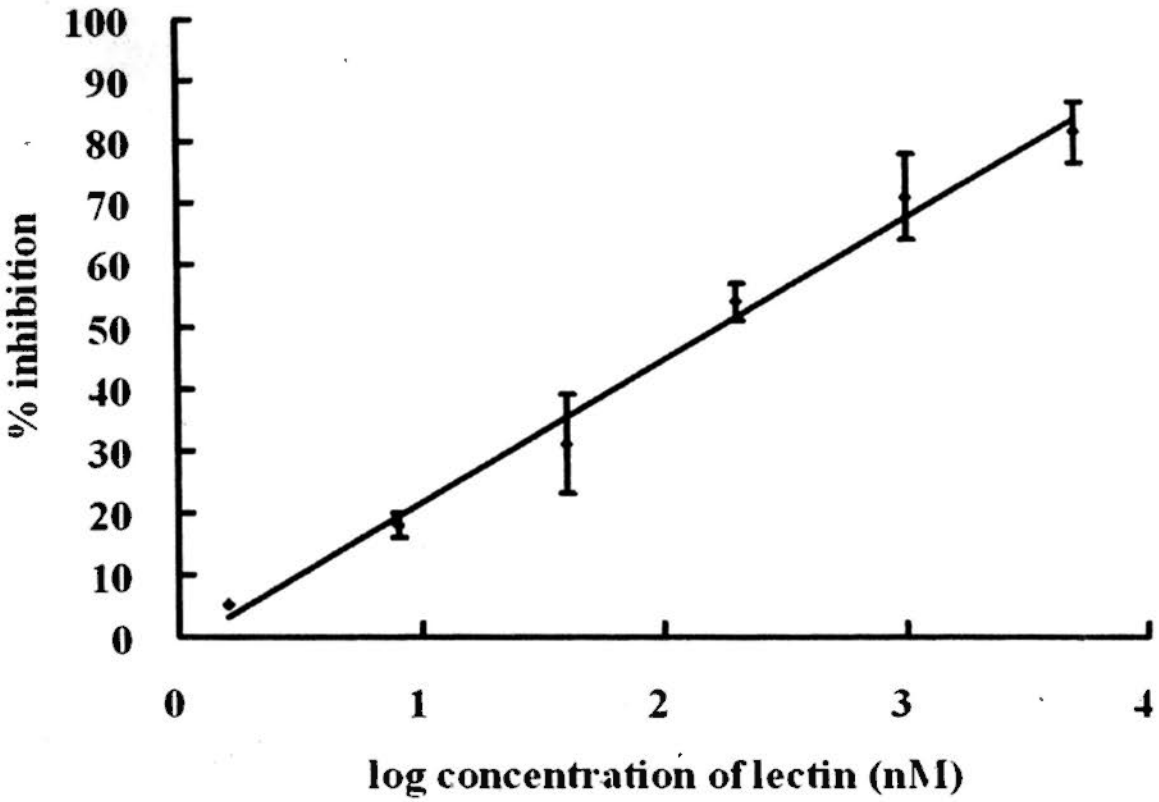


Figure 3.5. Inhibition of HIV-1 reverse transcriptase by *Hibiscus mutabilis* lectin. Percent inhibition compared to a control without the protein is indicated. Values are expressed as the mean ± SD (N=3).

Table 3.1. Protein yields from 420 g *Hibiscus mutabilis* seeds and specific hemagglutinating activities (ha) at different stages of purification of *Hibiscus mutabilis* lectin

Column	Fraction collected	Yield (mg)	Specific ha (unit/mg)	Total ha (unit)	Recovery of ha (%)	Purification fold of ha
-	Crude Extract	202	1025	207052	100	1
SP-Sepharose	S1	78	2108	164423	79	2.1
Superdex 75	S75-1	39	2632	102648	50	3.0
Superdex 200	S200-1	17	4000	68000	33	3.9

3.4. Discussion

To the best of our knowledge, there are only seven hexameric lectins reported in the literature, including those from *Axinella corrugata* (Dresch et al., 2008), *Ganoderma lucidum* (Thakur et al., 2007), *Helix pomatia* (Sanchez et al., 2006), *Ctenopharyngodon idellus* (Lam and Ng, 2002), *Trimeresurus albolabris* venom (Du et al., 2002), *Lactarius rufus* (Panchak and Antoniuk, 2007), and *Araucaria angustifolia* lectin (Datta et al., 1991). Even fewer examples of octameric lectins, e.g. *Anguilla japonica* lectin (Mistry et al., 2001), and only a single decameric lectin purified from *Araucaria angustifolia* (Datta et al., 1991) have been reported. All hexameric lectins reported are stable at around pH 4 to pH 8 and around 50 °C to 65 °C. The *H. mutabilis* lectin resembles them in pH stability and thermostability.

Many lectins manifest binding to one sugar (Sultan et al., 2009) or two sugars (Devi et al., 2009). In some cases, lectins can be inhibited by more than two sugars (Wang et al., 2002, Lam et al., 2009, Wang et al., 2000). It is worth noting that the *H. mutabilis* lectin is the first galactonic acid-binding lectin reported to date. This lectin is capable of agglutinating rabbit, but not rat or human red blood cells. As yet, there is no evidence that galactonic acid is present on the surface of rabbit erythrocytes. Thus, the lectin may also recognize some other sugars on the surface of rabbit erythrocytes that have not been tested in this study.

Some lectins are adsorbed on anion-exchangers (Sultan et al., 2009) or cation-exchangers (Sharma et al., 2009). Some are adsorbed on both types of exchangers (Xu et al., 2007). The *H. mutabilis* lectin is not adsorbed on a cation exchanger (SP-Sepharose), and it is tightly bound and cannot be dislodged from anion-exchangers (DEAE-cellulose and Q-Sepharose) by 2 M NaCl in 20 mM ammonium acetate buffer (pH 4.6). The purification scheme mainly depends on gel filtration, which is different from the previously reported lectins.

Information pertaining to the biological activities of hexameric lectins is meager. The *A. corrugata* lectin displays a chemotactic effect on rat neutrophiles (Dresch et al., 2008). The *C. idellus* lectin is mitogenic toward murine splenocytes and peritoneal exudate cells (Lam and Ng, 2002). There are no reports on the biological activities of other hexameric, octameric and decameric lectins.

The *H. mutabilis* lectin potently inhibited HIV-1 reverse transcriptase with an IC_{50} of 0.2 μM. Its activity is much stronger than that of other lectins which display an IC_{50} of 3 – 35 μM, including pinto bean lectin (Wong et al., 2006) and *Xerocomus spadiceus* lectin (Liu et al., 2004). Its potency is similar to that of *C. spinosa* lectin (Lam et al., 2009). The mechanism of inhibition is probably

protein-protein interaction, HIV-1 protease inhibits the homologous reverse transcriptase by protein-protein interaction (Böttcher and Grosse, 1997).

Not all lectins showed antiproliferative activity on tumor cells, e.g. *Rachycentron canadum* lectin (Ngai and Ng, 2007b). Some lectins exhibit antiproliferative or antitumor activity (Xu et al., 2007). The *H. mutabilis* lectin at 100 μM concentration produced only about 50 % inhibition of proliferation in HepG2 and MCF-7 cells. There are only a small number of reports on antifungal lectins, e.g. *Artocarpus genus* lectin can inhibit *Fusarium moniliforme* and *Saccharomyces cerevisiae* (Trindade et al., 2006). The *H. mutabilis* lectin is similar to that of most other lectins that are devoid of antifungal activity.

The N-terminal amino acid sequence of *H. mutabilis* lectin is ACVAPLDEAACAAAK. To date, no lectins have been reported with this partial sequence.

The subunits of a protein in the quaternary structure must be in non-covalent association (Devlin, 1997). The subunits in dimeric or tetrameric leguminous lectins comprise a flat six-stranded β-sheet and a curved seven-stranded β-sheet linked by loops of different lengths (Banerjee et al., 1994). The monomer of hexameric *Helix pomatia* agglutinin is composed of a six-stranded antiparallel β-sandwich. The

β-strands are linked and produce a hairpin at one extremity of the β-sandwich (Sanchez et al., 2006). The subunit arrangement of *H. mutabilis* lectin probably resembles that of hexameric *Helix pomatia* agglutinin.

All in all, the reported *H. mutabilis* lectin is unique in its high molecular mass, multimeric nature, sugar specificity, novel N-terminal amino acid sequence and extremely strong adsorption to anionic exchangers.

Chapter 4: Isolation and Characterization of a French Bean Hemagglutinin with Multiple Activities and Demonstration of its Apoptotic Pathway in Breast Cancer Cells

4.1. Introduction

Phaseolus vulgaris is a leguminous species with different cultivars. Numerous proteins have been purified from different cultivars of *Phaseolus vulgaris*. The main class is plant defense proteins, including trypsin inhibitors (Furuichi et al. 1993), aminopeptidases (Abdala et al., 1999), lectins (Ye et al., 2001b), chitinases (Ye and Ng, 2002), proteinases (Zakharov et al., 2004), antifungal peptides (Coda et al., 2008) and defensins (Games et al., 2008). Other functional proteins, like alpha-amylase inhibitor (Yamaguchi, 1991), alcohol dehydrogenase (Grima-Pettenati et al., 1994), beta-galactosidase (Biswas et al., 2003), phosphatase (Tejera García et al, 2004), allantoinase (Raso et al., 2007b), and allantoate-degrading enzyme (Raso et al., 2007a), have also been investigated.

Lectins and hemagglutinins have been investigated for potential application as antiviral (Balzarini et al., 1992) and antitumor agents. Phytohaemagglutinin, pokeweed mitogen, soybean agglutinin, and wheat germ agglutinin restrict tumour growth and improve the life expectancy of the host (Ganguly and Das, 1994). Galectins play important roles in immune and inflammatory responses, tumour

development and progression, neural degeneration, atherosclerosis, diabetes, and wound repair. They may be applied as therapeutic agents for inflammatory diseases, cancers, etc (Yang et al., 2008).

In order to increase its potential of clinical application, the detailed apoptotic mechanism should be studied. There are two main pathways of apoptosis, the death receptor-mediated pathway and mitochondrial pathway. Several plant lectins can induce tumor cell apoptosis (Liu et al., 2008b, Lam et al., 2009, Seifert et al., 2008, Lei and Chang, 2007). However, there is a scarcity of reports pertaining the pathway involved (Gastman et al., 2004, Brandt et al., 2008, Khil et al., 2007, Liu et al., 2008b, Boleti et al., 2008).

There are very few reports in the literature about induction of apoptosis in MCF-7 cells by hemagglutinins. No information about the detailed apoptotic pathway involved is available. Thus, the objective of the present study was to isolate a hemagglutinin from *Phaseolus vulgaris* cultivar "French bean number 35", and to investigate details of the apoptotic pathway in MCF-7 cells it induced. The results revealed that the apoptosis involves a death receptor-mediated pathway. The investigation of the apoptotic pathway in MCF-7 cells makes it possible in clinical application as an anti-tumor drug.

4.2. Materials and Methods

4.2.1. Purification of hemagglutinin

Dried seeds of *Phaseolus vulgoris* cv. "French bean number 35" (100 g) were purchased from Vegetable Research Institute, Guangdong Academy of Agriculture, Guangdong, China. They were extracted by homogenization in distilled water (10 ml/g seeds). Following centrifugation at 20000 g for 30 min at 4 °C, Tris-HCl buffer (2 M, pH 7.4) was added to the supernatant until the final concentration of Tris reached 20 mM. The supernatant was then applied on a 5 cm x 10 cm column of Blue-Sepharose (GE Healthcare). Unadsorbed proteins were eluted with 20 mM Tris-HCl buffer (pH 7.4). Adsorbed proteins were eluted with 0.5 M NaCl added to the 20 mM Tris-HCl buffer. The eluted fraction was taken, dialyzed, and then subjected to chromatography on a 5 cm x 10 cm column of Q-Sepharose (GE Healthcare). After removal of unadsorbed proteins by elution with 20 mM Tris-HCl buffer (pH 7.4), adsorbed proteins were first eluted with a gradient of 0 M to 0.5 M NaCl added to the 20 mM Tris-HCl buffer, and finally with 1 M NaCl in 20 mM Tris-HCl buffer (pH 7.4). The fraction eluted with the 0 – 0.5 M NaCl gradient was saved, dialyzed against distilled water, and lyophilized before chromatography on a gel filtration column of Superdex 75 10/300 GL (GE Healthcare). The first fraction collected represented purified hemagglutinin.

4.2.2. Molecular mass determination using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, gel filtration, and N-terminal amino acid sequencing were described in Section 2.2.2.

4.2.3. Assay for hemagglutinating activity was described in Section 2.2.4.

4.2.4. Inhibition of hemagglutinin-induced hemagglutination by carbohydrates was described in Section 2.2.5.

4.2.5. Inhibition of lectin-induced hemagglutination by metal ions was described in Section 3.2.5.

4.2.6. Effects of temperature and pH on hemagglutinin-induced hemagglutination were described in Section 2.2.6.

4.2.7. Assay of antifungal activity was decribed in Section 2.2.7.

4.2.8. Assay of HIV-1 reverse transcriptase inhibitory activity was described in Section 2.2.8.

4.2.0. Assay of antiproliferative activity was described in Section 2.2.9.

4.2.10. Annexin-V and propidium iodide (PI) staining was described in Section 2.2.10.

4.2.11. Annexin-V and PI staining of z-IETD-fmk treated MCF-7 cells

MCF-7 cells were treated with 1 μM caspase-8 inhibitor z-IETD-fmk (Biovision) for 16 hours. Different concentrations of hemagglutinin were then added to the z-IETD-fmk-treated cells. After incubation for 24 hours, the cells were analyzed by a FASCsort flow cytometer.

4.2.12. Measurement of mitochondrial transmembrane potential by JC-1 staining

The fluorescent dye JC-1 was employed for the determination of mitochondrial transmembrane potential (Cheung et al., 2005). Briefly, cells (5×10^5) were treated with various concentrations of the hemagglutinin for 24 hours before harvesting. They were then washed twice with PBS. The cell pellets were resuspended in 500 μl plain RPMI medium containing 2.5 μg/ml JC-1 dye, and left in the dark for 15 min at 37 °C. The mitochondrial depolarization patterns of the cells were observed by using FACSort flow cytometer.

4.2.13. Cell cycle analysis

Cells (5×10^5/ well) were seeded in 6-well plates and incubated in the presence of various concentrations of the hemagglutinin for 24 hours. They were then harvested, washed with PBS, and fixed in 70 % ice-cold ethanol at 4 °C for 2

hours. Afterwards, the cells were washed twice with PBS and resuspended in 1 ml stain solution (20 μg/ml PI in PBS, containing 1% Triton X-100 and 10 μg/ml RNase A) at 37 °C in the dark for 30 min. Fluorescence emitted from the PI-DNA complex was examined with flow cytometry (Cheung et al., 2007).

4.2.14. DNA fragmentation detection

Cells collected after hemagglutinin treatment for 24 hours were resuspended in cell lysis buffer, and incubated at 37 °C for 2 hours. Then, saturated NaCl solution was added. After vortexing and centrifugation, DNA was precipitated with ice-cold absolute ethanol. After centrifugation, the pellet obtained was rinsed with 75% ethanol, air-dried, and dissolved in buffer containing RNase A. Samples were then analyzed by agarose gel electrophoresis (Ho et al., 2006).

4.2.15. Subcellular localization of bimane-labeled (fluorescent) hemagglutinin in MCF-7 cells

The lyophilized hemagglutinin was dissolved in 2 mM MES buffer (pH 5.0) in the fluorescent dye bimane amine (final concentration 10 mM) (Invitrogen) and the linker EDC (carbodiimide) (final concentration 2mM) (Invitrogen). The mixture was incubated at room temperature for 2 hours with gentle stirring before centrifugation (10 min, 14000 rpm) to remove any precipitated protein. An Amicon (5000 molecular weight cutoff) was used to remove salts, unbound bimane amine, and EDC

from the labeled protein (van der Weerden et al., 2008). Bradford protein assay was used to determine protein concentration. The MCF-7 cells (60000 in 2 ml RPMI medium) were seeded in glass bottom culture dish and incubated with 15 μM labeled hemagglutinin for 24 hours. MCF-7 cells without hemagglutinin-treatment acted as negative control. Cells were washed with PBS twice before analyzed on a SP5 confocal microscope (Leica Microsystems GmbH, Heidelberg, Germany) using an excitation wavelength of 388 nm and blue fluorescence images was captured.

4.2.16. Western blot study of whole-cell lysate

Cells (1×10^7) were treated with hemagglutinin for 24 hours. The cells were disrupted in 200 μl lysis buffer (4.9 mM $MgCl_2$, 100 mM $NaVO_3$, 10% Triton X-100, 100 mM PMSF, 2.1 mg/ml aprotinin, 1 mg/ml leupeptin in PBS) on ice for 15 min. The lysates were boiled for 10 min and centrifuged (14000 g, 10 min). The supernatants were saved. Protein concentration was quantified by using the Bradford assay. The supernatant (30 μg protein) was mixed with the loading dye, electrophoresed on a 12.5% SDS-PAGE, and then transferred to a PVDF membrane. The membranes were incubated with specific primary antibodies [mouse monoclonal anti-human β-actin and anti-Bcl-2 (Santa Cruz Biotechnology), anti-procaspase-9 (Calbiochem), anti-p53 (Invitrogen) and rabbit polyclonal anti-procaspase-8, anti-FAS, anti-Bax, anti-Bid, and anti-Bad (Santa Cruz

Biotechnology), anti-Lamin-A/C (Abcam) antibodies overnight at 4 °C. Further incubation with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology)] for 90 min at 4 °C was then carried out. Detection was performed using an enhanced chemiluminescence (ECL) kit (GE Healthcare) (Tang et al., 2006).

4.1.17. Western blot study of mitochondrial and cytosolic fractions

Hemagglutinin-treated cells (1×10^7) were harvested, washed with PBS, and resuspended in 50 µl buffer A (250 mM sucrose containing 75 mM NaCl, 1 mM NaH_2PO_4, 8 mM Na_2HPO_4, 100 mM phenylmethylsulfoxyl fluoride (PMSF), 2.1 mg/ml aprotinin, 1 mg/ml leupeptin, and 0.5 mg/ml digitonin in PBS). The cell suspensions were vortexed and centrifuged (2000 g, 1 min). The supernatants obtained constituted the cytosolic fraction. The pellets were resuspended in 50 µl buffer B (250 mM sucrose, 75 mM NaCl, 1 mM NaH_2PO_4, 8 mM Na_2HPO_4, 100 mM PMSF, 2.1 mg/ml aprotinin, 1 mg/ml leupeptin, 5 mg/ml digitonin in PBS), and centrifuged (2000g, 1 min), and the supernatants were saved as the mitochondrial fraction (Tang et al., 2006). Western blot analysis was conducted using specific primary antibodies (mouse monoclonal anti-cyt c antibody; mouse monoclonal antihuman β-actin, and anti-(Apoptosis-Inducing Factor) AIF antibodies, Santa Cruz Biotechnology).

4.3. Results

The French bean extract was resolved on Blue-Sepharose into two fractions: a slightly larger unadsorbed fraction B1 without hemagglutinating activity and a slightly smaller adsorbed fraction B2 with hemagglutinating activity (Figure 4.1). Fraction B2 was separated on Q-Sepharose into a very small unadsorbed fraction Q1, a large adsorbed fraction Q2, and a very small adsorbed fraction Q3. Hemagglutinating activity was confined to fraction Q2 (Figure 4.2). Fraction Q2 was subsequently separated on Superdex 75 10/300 GL into a major fraction S1 with hemagglutinating activity and a minor fraction S2 without activity (Figure 4.3). Fraction S1 represented purified hemagglutinin as witnessed by a single 32-kDa band in SDS-PAGE (Figure 4.4).

The yields and hemagglutinating activities at different stages of purification of French bean hemagglutinin are summarized in Table 4.1. Its N-terminal sequence, ATETYSAFQRFCETNIFIQR, resembled those of other *Phaseolus* hemagglutinins (Table 4.2). There was no inhibition when the following sugars were tested up to 100 mM: D-glucosamine, mannitol, D-xylose, sucrose, D-fucose, D-raffinose, α-lactose, D-fructose, L-arabinose, D-galacturonic acid, D-galactose, D-mannose, D-glucuronic acid, D-glucose and D-sorbitol. Of the various salts tested, only calcium chloride restored the activity of the hemagglutinin. The hemagglutinating activity of the

hemagglutinin was stable in the pH range 6 – 8. About 70 % activity was retained in pH 2 – 5 and pH 8 – 12. The activity was completely eliminated at pH 1, 13 and 14. The activity was stable in the temperature range 0 °C – 50 °C, reduced to half at 70 °C, and demolished at 80 °C.

The hemagglutinin inhibited HIV-1 reverse transcriptase with an IC_{50} of 2 μM. It inhibited mycelial growth in *Valsa mali* with an IC_{50} of 10 μM (Figure 4.5) but there was no effect on *Mycosphaerella arachidicola, Fusarium oxysporum, Helminthosporium maydis,* and *Rhizoctonia solani.* It reduced the viability of MCF-7 cells and HepG2 cells, with an IC_{50} of 2 μM and 100 μM, respectively. By comparison, the positive control doxorubicin exhibited an antiproliferative activity toward these tumor cells with an IC_{50} of 5 μM and 10 μM, respectively. French bean hemagglutinin had no antiproliferative effect on normal embryonic liver WRL68 cells (Figure 4.6). The comparison of pharmacological activities of French bean hemagglutinin to various positive controls used in this study, including doxorubicin, nystatin and *B. campestris* lipid transfer protein, is summarized in Table 4.3.

For investigating the mode of cell death, cell cycle analysis was performed at different concentrations of the hemagglutinin. When compared to the control, the distribution of cells in various phases of the cell cycle was affected by treatment with the hemagglutinin for 24 hours: in the G0/G1 phase (with an increase from 42.5 % in

control cells to 55.6 % when 45 μM hemagglutinin was added) and in the G2/M phase (rising from 14.8 % in control cells to 30.1 % upon addition of 45 μM hemagglutinin). G0/G1 phase and G2/M phase arrests were discerned in a dose-dependent manner (Figure 4.7).

To corroborate that cell death induced by the hemagglutinin was associated with apoptosis, phosphatidylserine externalization onto the cell surface was investigated by using annexin V-FITC/ PI staining. The results disclosed that the proportion of annexin-V stained cells representing both the early and late apoptotic cells rose as the concentration of hemagglutinin applied was raised (Figure 4.8a). At low concentrations of hemagglutinin (0.5, 1.5 and 5 μM), a substantial percentage of cells was already present in the early phase of apoptosis. Exposure to higher concentrations (15 and 45 μM) of the hemagglutinin ensued in a shift of the cell population to the late apoptotic/necrotic stage. The table in Figure 4.8a shows that the percentage of MCF-7 cells undergoing apoptosis increased as the hemagglutinin concentration increased.

Early cellular apoptosis is escorted by disruption of mitochondrial membrane, resulting in a rapid dissipation of the electrochemical gradient (Ly et al., 2003). The effect of the hemagglutinin on mitochondrial transmembrane potential of MCF-7 cells was studied by using a mitochondrion-specific dye, JC-1. Flow cytometric

analysis revealed that more cells became susceptible to mitochondrial membrane depolarization when the hemagglutinin concentration was elevated (Figure 4.9). A remarkable attenuation (39.3 %) of mitochondrial transmembrane potential occurred in cells exposed to 45 μM hemagglutinin.

The subcellular localization of bimane-labeled (fluorescent) hemagglutinin was investigated. The labeled hemagglutinin was not detected on the cell surface or inside the cells under a SP5 confocal microscope (not shown). The results indicated that the hemagglutinin was not taken up by the MCF-7 cells.

As the concentration of the hemagglutinin increased, there was a dose-dependent increase in the expression of FAS, truncated Bid, p53, Bak, cytosolic cytochrome c, cytosolic AIF, and truncated Lamin A/C. In contrast, there was a dose-dependent reduction in the expression level of anti-apoptotic factor, Bcl-2, and the pro-forms of upstream initiator caspases, pro-caspase-8 and pro-caspase-9. The expression of Bax and total cytochrome c and total AIF remained unaltered (Figure 4.10).

The percentage of cells undergoing apoptosis/ necrosis underwent a decline upon addition of the caspase-8 inhibitor z-IETD-mfk. The results were summarized in the table in Figure 4.8b. After exposure to z-IETD-mfk, about 60 % decrease in the percentages of hemagglutinin-treated MCF-7 cells undergoing apoptosis/

necrosis was observed, indicating that Fas and caspase-8 are involved in the apoptotic pathway.

DNA fragmentation was not observed in hemagglutinin-treated MCF-7 cells (not shown).

A proposed pathway of the apoptosis induced by French bean hemagglutinin is presented in Figure 11. Accompanying the dose-dependent increase in the expression of p53, morphological changes were observed (Figure 4.12).

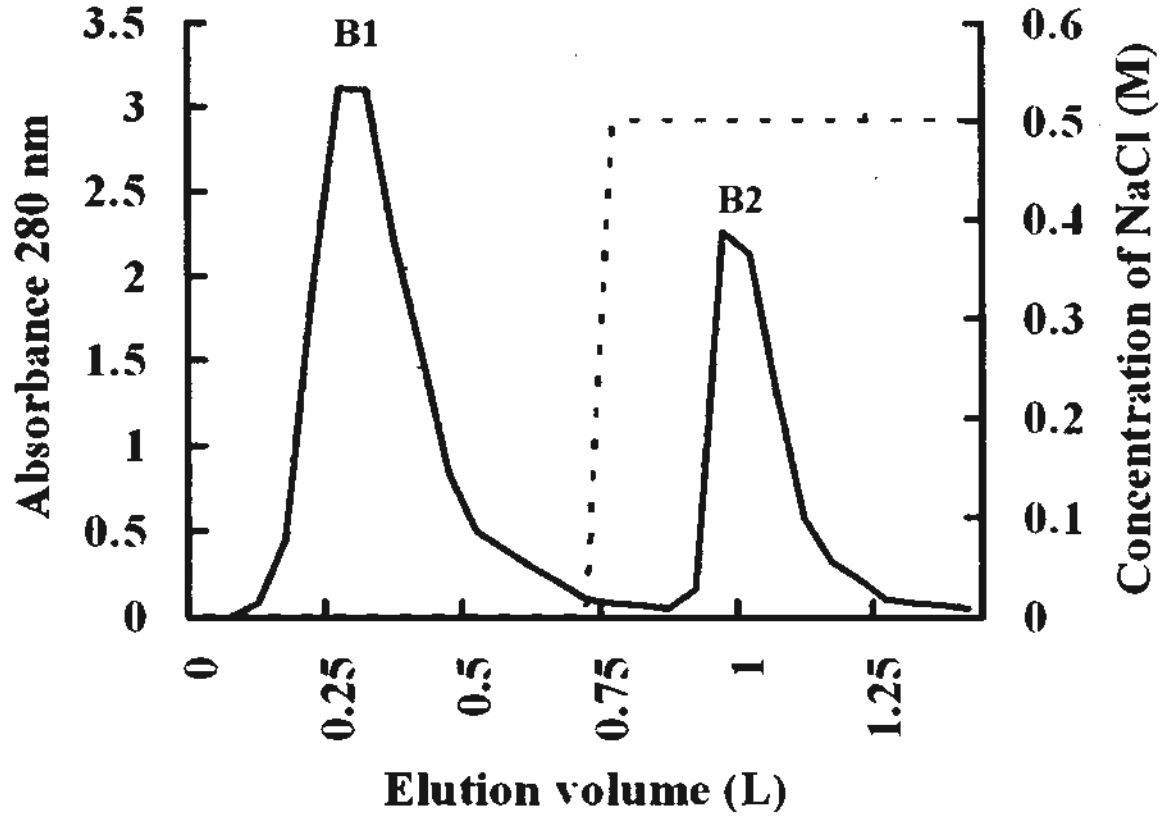


Figure 4.1. Affinity chromatography of French bean seed extract on Blue-Sepharose column (5 cm x 10 cm).

The column had been equilibrated with 20 mM Tris-HCl buffer (pH 7.4) before application of sample. The dotted lines indicate the use of 0.5 M NaCl in the starting buffer to elute fraction B2. Flow rate = 10 ml/min. Hemagglutinating activity was detected only in fraction B2.

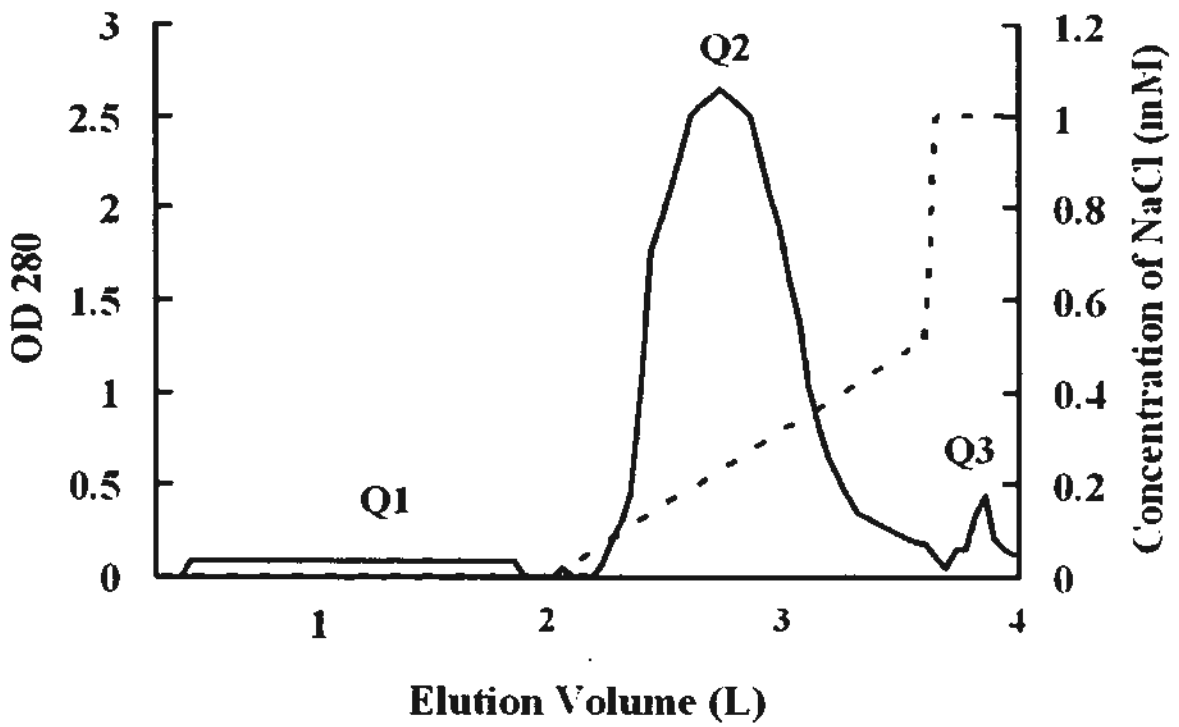


Figure 4.2. Anion-exchange chromatography of fraction B2 on a Q-Sepharose column (5 cm x 10 cm).

The dotted line indicates the use of a linear 0 – 0.5M NaCl gradient to elute fraction Q2 and the use of 1 M NaCl to elute fraction Q3. Flow rate = 10 ml/min. Hemagglutinating activity was detected only in fraction Q2.

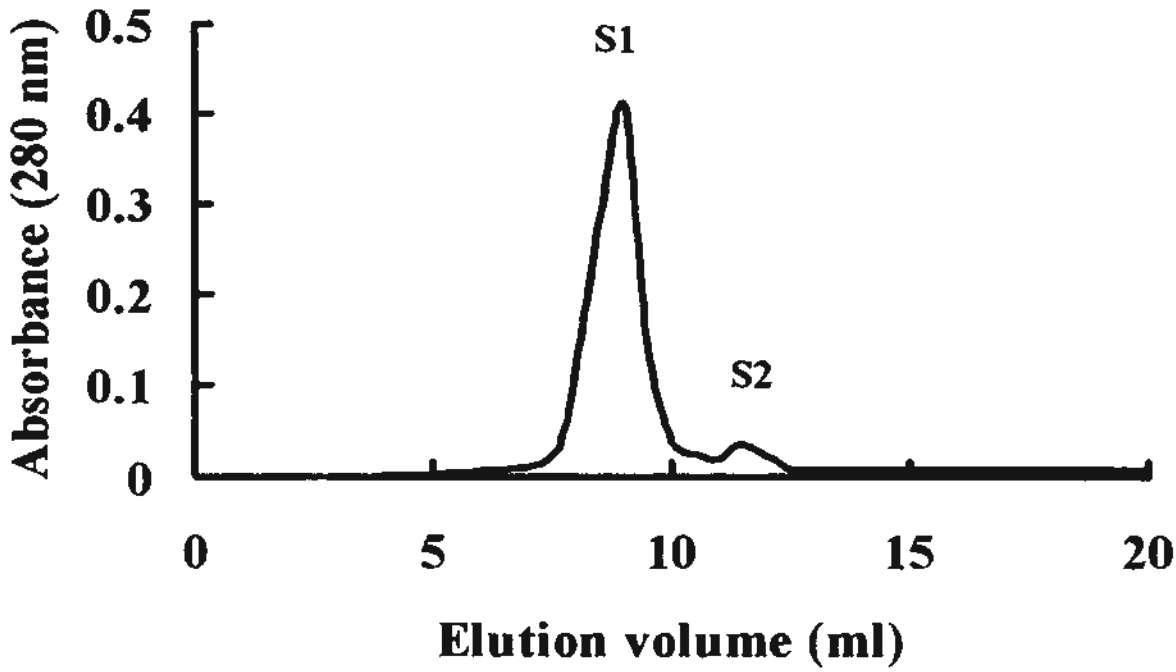


Figure 4.3. Gel filtration of fraction Q2 on a Superdex 75 10/300 GL column.

The running buffer was 200 mM NH_4HCO_3 (pH 7.2). Flow rate = 0.5 ml/min.

Hemagglutinating activity was detected only in fraction S1.

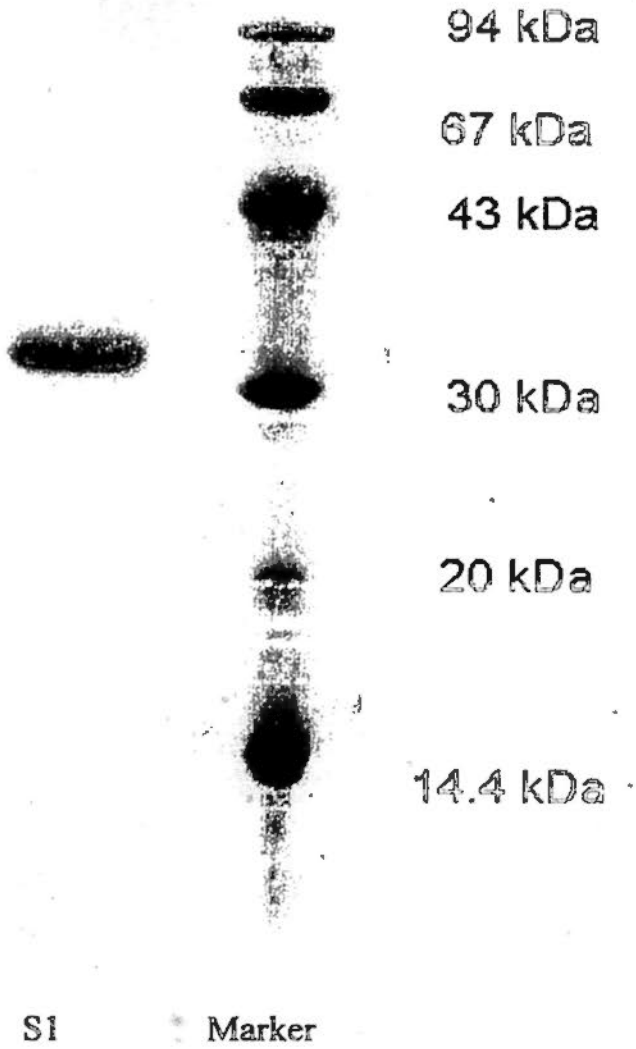


Figure 4.4. SDS-polyacrylamide gel electrophoresis. Left lane: Fraction S1 representing purified French bean hemagglutinin. Right lane: Molecular mass markers from GE Healthcare. From top downward: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14.4 kDa).

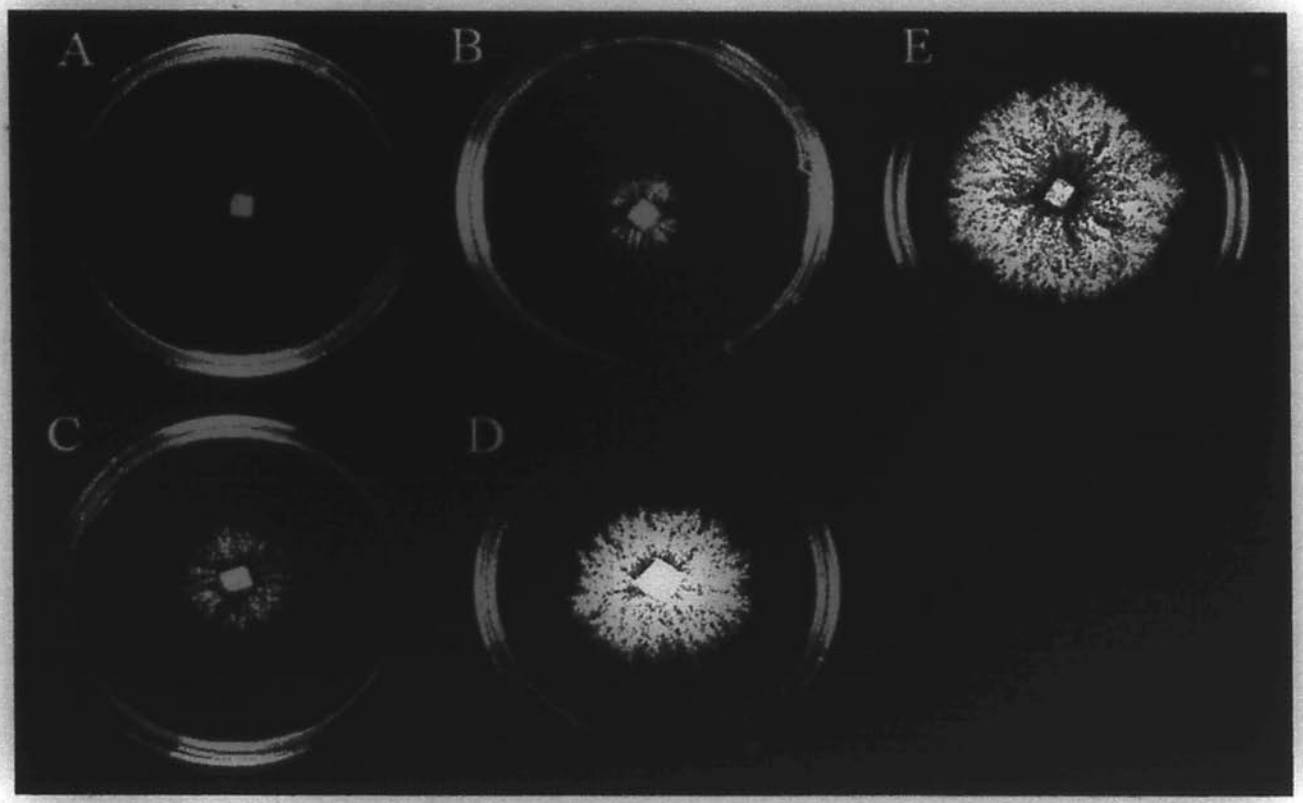


Figure 4.5. Antifungal activity of French bean hemagglutinin toward *Valsa mali*.

A: 90 μM hemagglutinin, B: 30 μM hemagglutinin. C: 10 μM hemagglutinin. D: 3.3 μM hemagglutinin. E: buffer control.

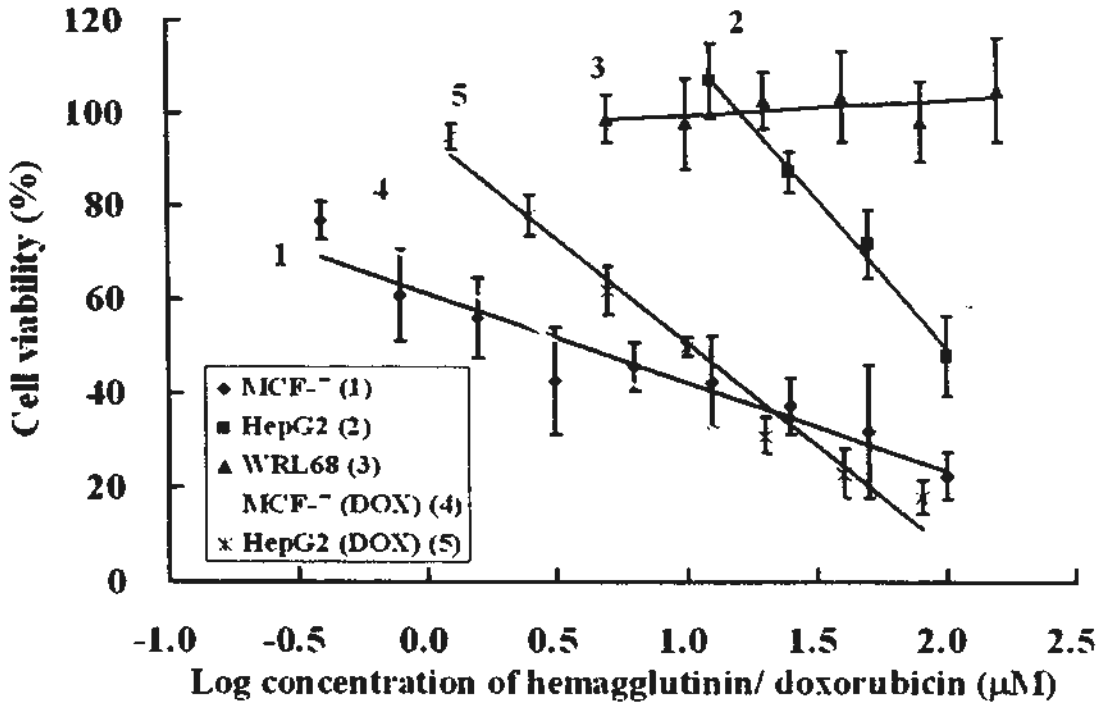
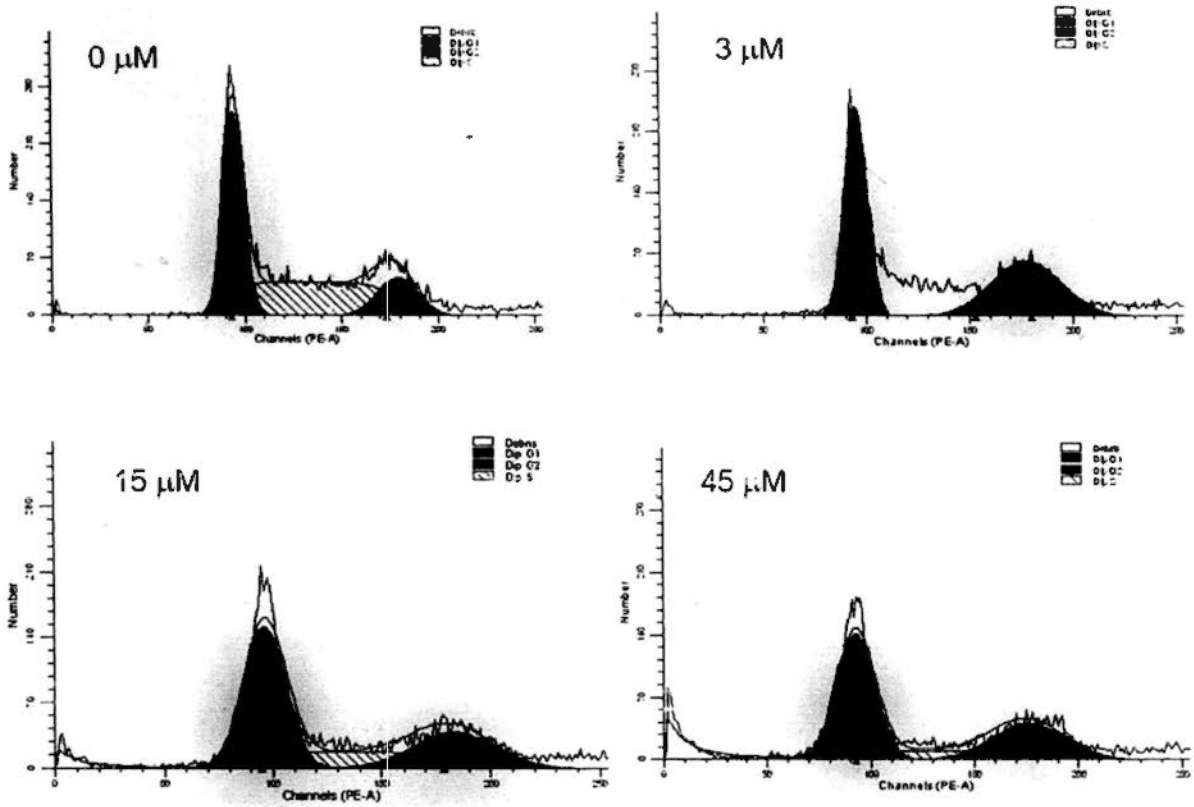


Figure 4.6. Effects of French bean hemagglutinin on viability of breast cancer MCF-7 cells, hepatoma HepG2 cells and normal embryonic liver WRL68 cells.

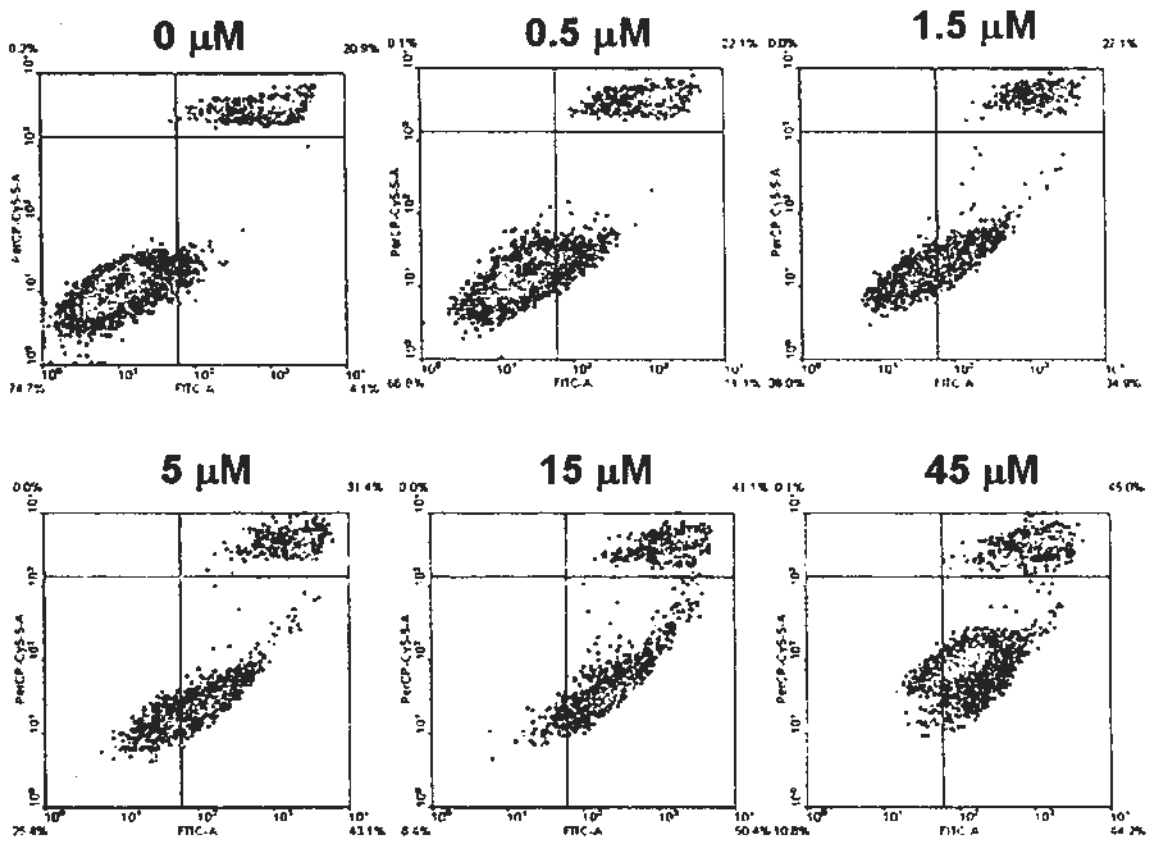
The viability of MCF-7, HepG2 and WRL68 cells, after incubation for 48 hours in the presence of various concentrations of French bean hemagglutinin or doxorubicin (DOX, as positive control), was determined as described in section 2.2.9. Viability of cells in RPMI medium only was taken as 100 %. Values are expressed as mean ± SD (N = 3).



Hemagglutinin (μM)	G0/G1 (%)	S (%)	G2/M (%)
0	42.5 ± 2.8	42.7 ± 4.6	14.8 ± 1.6
3	43.6 ± 3.8	37.5 ± 3.3	18.9 ± 2.5
15	52.5 ± 2.9	21.9 ± 3.0	25.6 ± 3.3
45	55.5 ± 3.2	14.5 ± 1.4	30.1 ± 4.0

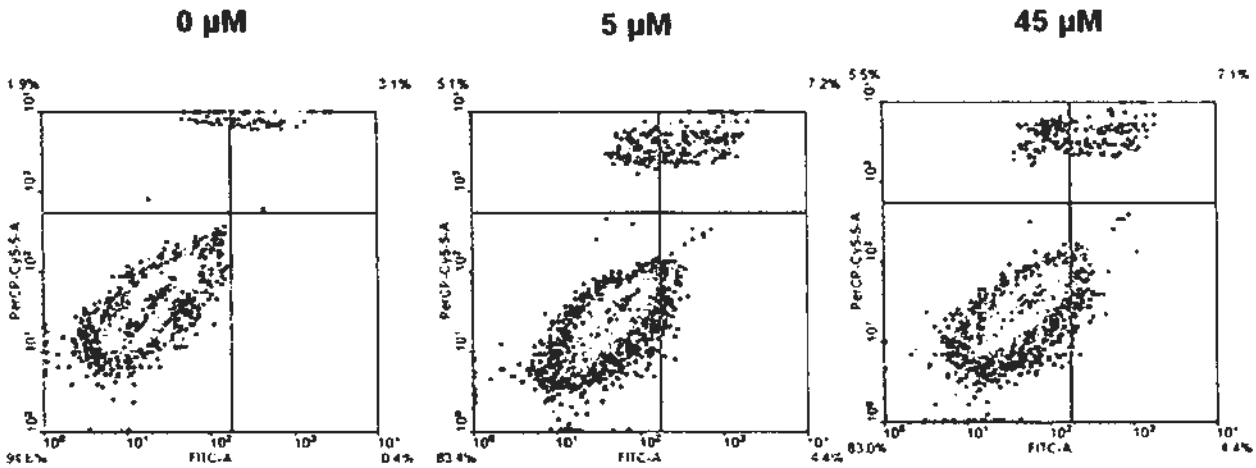
Figure 4.7. Cell cycle analysis of MCF-7 cells after hemagglutinin treatment. Cells were treated with French bean hemagglutinin (0, 3, 15 and 45 μM) for 24 hours. Cells were harvested and stained with propidium iodide and subjected to flow cytometric analysis. The table summarizes the percentages of cells in each phase of the cell cycle after hemagglutinin treatment. Values are expressed as mean ± SD (N = 3). The figure shows results of one of the three experiments.

(a)



Concentration of hemagglutinin (µM)	Normal cells	Cells undergoing early apoptosis	Cells undergoing late apoptosis/ necrosis
0	74.7 % ± 2.7 %	4.1 % ± 0.6 %	20.9 % ± 1.4 %
0.5	66.8 % ± 3.1 %	11.1 % ± 0.9 %	22.1 % ± 1.6 %
1.5	38.0 % ± 1.9 %	34.9 % ± 2.3 %	27.1 % ± 2.1 %
5	25.4 % ± 1.2 %	43.1 % ± 3.1 %	31.4 % ± 2.2 %
15	8.4 % ±0.8 %	50.4 % ± 4.1 %	41.1 % ± 1.6 %
45	10.8 % ± 0.9 %	44.2 % ± 3.3 %	45.0 % ± 2.5 %

(b)



Concentration of hemagglutinin (µM)	% of apoptotic / necrotic cells in absence of caspase-8 inhibitor	% of apoptotic / necrotic cells in presence of caspase-8 inhibitor
0	25.0 ± 2	3.5 ± 0.3
5	74.5 ± 5.3	11.6 ± 0.8
45	89.2 ± 5.8	11.5 ± 1.0

Figure 4.8. (a) Annexin V-FITC/ PI staining of MCF-7 cells treated with different concentrations of French bean hemagglutinin (0, 0.5, 1.5, 5, 15 and 45 μM) for 24 hours. Cells collected were subjected to staining and then analyzed with a flow cytometer. The table below Figure 4.8a summarizes the percentages of cells at each stage of apoptosis. Values are expressed as mean ± SD (N = 3). The results shown here were from one of three experiments. (b) Annexin V-FITC/ PI staining of z-IETD-mfk treated MCF-7 cells after incubation in the presence of different concentrations of hemagglutinin. The percentages of cells undergoing apoptosis/ necrosis are summarized in the table below Fig 4.8b. The decrease in the percentages of apoptotic/ necrotic cells after z-IETD-mfk treatment and incubation with hemagglutinin indicated FAS and caspase-8 are involved in the apoptotic pathway. The results represented one of three experiments.

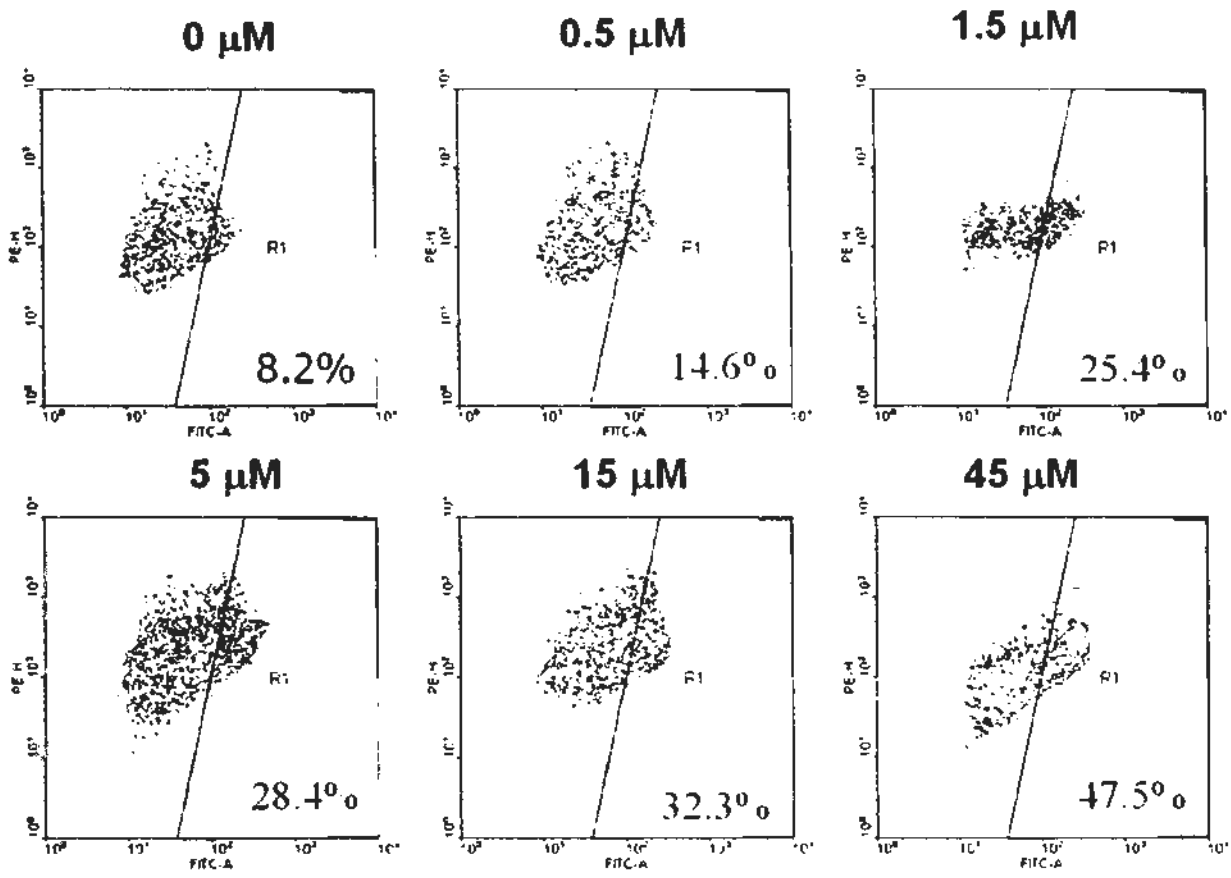


Figure 4.9. Depolarization of mitochondrial membrane in hemagglutinin-treated MCF-7 cells. Cells were incubated in the presence of different concentrations of French bean hemagglutinin (0, 0.5, 1.5, 5, 15, 45 μM) for 24 hours. Cells collected were stained with JC-1 dye. Fluorescence emission was measured by a flow cytometer. Region R1 corresponds to the percentage of cells with depolarized mitochondrial membrane. The results represented one of three experiments.

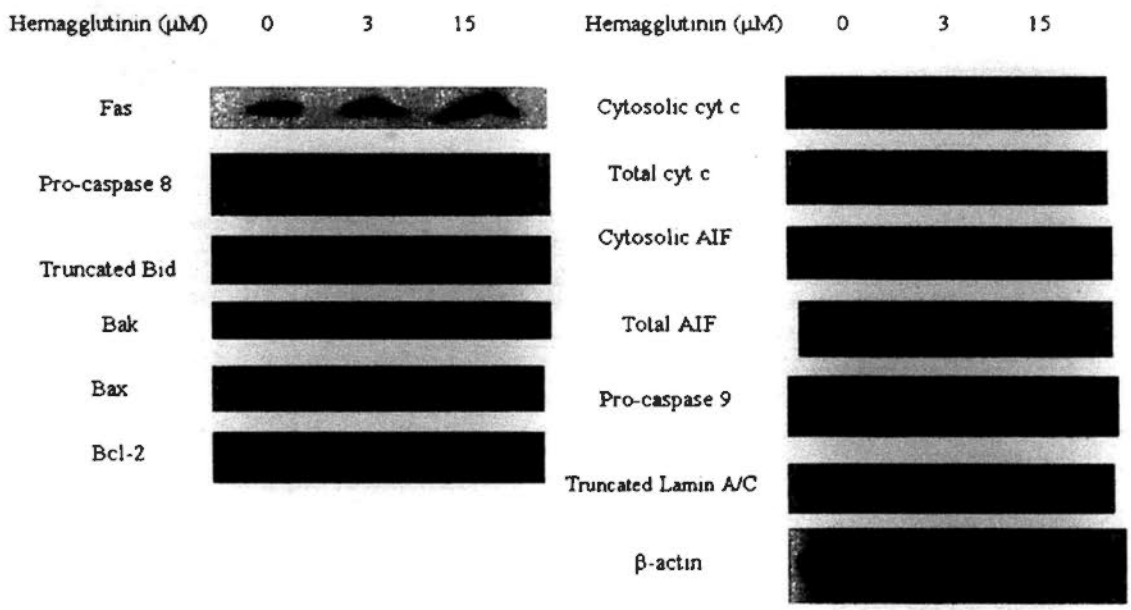


Figure 4.10. Western blot analysis. β-actin was used as an internal control. The increased expression level of Fas indicated that Fas ligands are the death receptor, which activated caspase 8. Truncation of Bid, together with the increase of Bak, and down-regulation of Bcl-2, prompted mitochondrial release of cyt c and AIF. Caspase 9 was then activated, causing the truncation of Lamin A/C, which targeted cell shrinkage and membrane blebbing, finally causing the cells to undergo apoptosis. The figures represented one of three experiments.

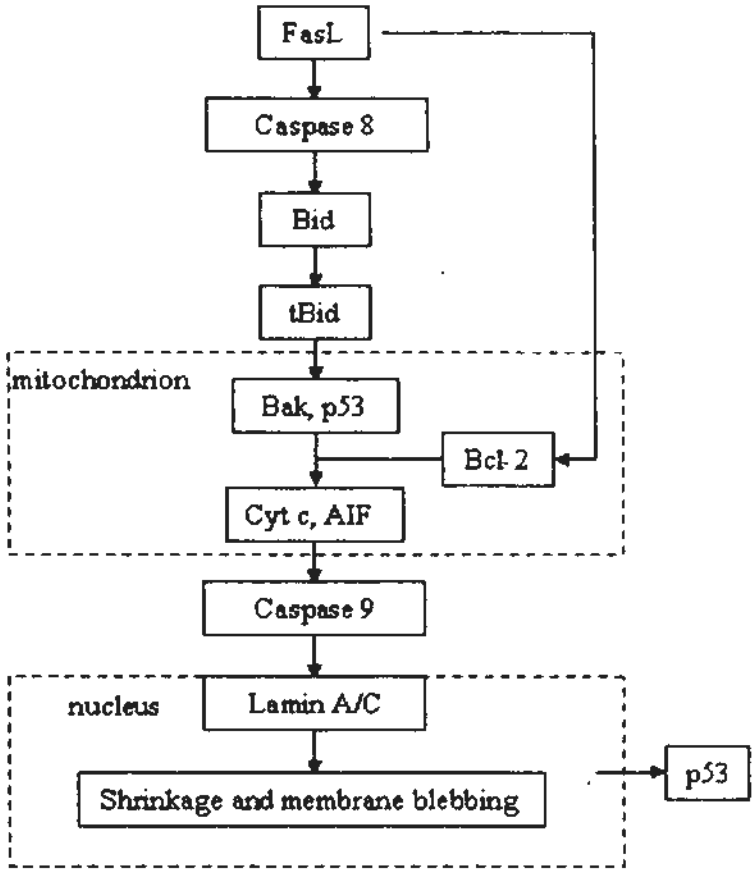


Figure 4.11. The proposed apoptotic pathway in MCF-7 cells induced by French bean hemagglutinin. The pathway is deduced from the results of Western blot and based on the flowchart apoptotic pathway which can be downloaded from Cell Signaling Technology website (www.cellsignal.com).

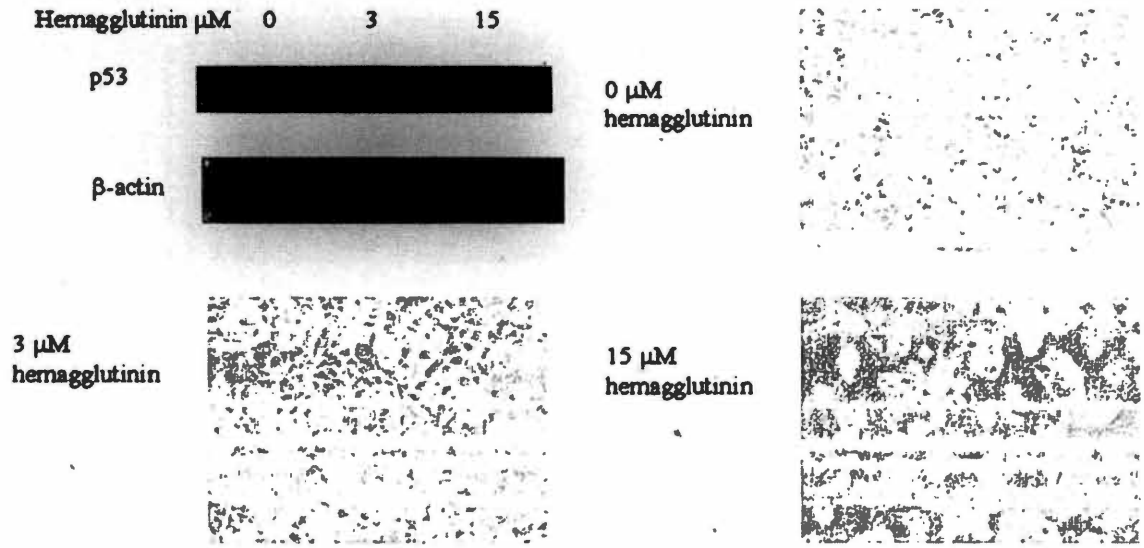


Figure 4.12. Western blot analysis was performed with mouse monoclonal anti-p53. β-actin was used as an internal control. Increased level of p53 after treatment with 15 μM hemagglutinin induced morphological changes in MCF-7 cells. The results represented one of three experiments.

Table 4.1. Protein yields and hemagglutinating activities (ha) of French bean hemagglutinin at different stages of purification from 100 g French bean seeds.

Purification Stages	Fraction collected	Yield (g)	Specific ha (titer/g)	Total ha (titer)	Recovery of ha (%)	Fold of purification
After Extraction	-	5.0	1.0×10^7	5.0×10^7	100	1
After Blue-Sepharose	B2	1.6	2.2×10^7	3.5×10^7	70	2.2
After Q-Sepharose	Q2	1.4	2.4×10^7	3.3×10^7	60	2.4
After Superdex 75	S1	1.1	2.7×10^7	3.0×10^7	54	2.7

Table 4.2. N-terminal amino acid sequence of French bean hemagglutinin.

Hemagglutinin	N-terminal Sequence	% Identity
French Bean 35 hemagglutinin	ATETYSAFQRFCETNLILQR	100
Phaseolus vulgaris cv. (Anasazi Beans)	ASETSFSFQRFVETNLILQR	75
Phaseolus coccineus lectin (22-41)	ASETSFSFDRFNETNLILQU	65
Phaseolus acutifolius lectin (25-44)	ANDISFNFQRFNETNLILQG	60

Table 4.3. Comparison of biological potencies of French bean hemagglutinin, doxorubicin, nystatin and *Brassica campestris* lipid transfer protein.

	French bean hemagglutinin (IC_{50})	Doxorubicin (IC_{50})	Nystatin (IC_{50})	*B. campestris* lipid transfer protein (IC_{50})
Antiproliferative activity against HepG2 cells	100 ± 15	10 ± 3.3	Not determined	7.3 ± 3.2
Antiproliferative activity against MCF-7 cells	2.0 ± 0.3	5.0 ± 2.1	Not determined	40 ± 2.9
Antifungal activity against *Valsa mali*	10 ± 1.3	ND	12.3 ± 2.1	Not determined
Inhibitory activity against HIV-1 reverse transcriptase	2.0 ± 0.2	Not determined	Not determined	5.2 ± 1.4

4.4. Discussion

French bean hemagglutinin isolated in the present investigation displays an N-terminal sequence similar to those of hemagglutinins from other *Phaseolus vulgaris* cultivars and *Phaseolus* species. Its molecular weight closely resembles those of hemagglutinins/ lectins from other *Phaseolus vulgaris* cultivars, such as dark red kidney bean lectin (Xia and Ng, 2006), red kidney bean lectin (Ye et al., 2001b), and pinto bean lectin (Wong et al., 2006). French bean hemagglutinin has only moderate pH stability, i.e. stability from pH 6 – 8, and thermostability at temperatures up to 50 °C. These characteristics were similar to that of most hemagglutinins (Leung et al., 2008).

The French bean hemagglutinin is adsorbed on Blue-Sepharose, this chromatographic behavior is same as other lectins/ hemagglutinins from different cultivars of *Phaseolus vulgaris*. On the other hand, French bean hemagglutinin is adsorbed on an anion-exchanger, which is similar to that of pinto bean lectin (Wong et al., 2006), haricot bean lectin (Wong and Ng, 2005a) and flageolet bean lectin (Xia and Ng, 2005). In contrast, some lectins are adsorbed on cation-exchangers but not on anion-exchangers, e.g. dark red kidney bean lectin (Xia and Ng, 2005), red kidney bean lectin (Xia and Ng, 2006) and anasazi bean lectin (Sharma et al., 2009).

French bean hemagglutinin resembles hemagglutinins of other cultivars of *P. vulgaris* in molecular mass and number of subunits. Antiproliferative activity, HIV-1 reverse transcriptase inhibitory activity, and antifungal activity have only been demonstrated for hemagglutinins/ lectins of only some cultivars. (Table 4.4)

To date only several lectins or hemagglutinins have been reported with antifungal activity. For example, red kidney bean lectin inhibits *F. oxysporum, Coprinus comatus,* and *R. solani* (Ye et al., 2001b). The spectrum of antifungal activity of French bean hemagglutinin (against *V. mali*, but not against others) is similar to *C. spinosa* lectin (Lam et al., 2009) but distinct from those of other antifungal lectins.

The ability of French bean hemagglutinin to inhibit proliferation of tumor cells is distinct from other lectins. Only a few lectins, e.g. *C. spinosa* lectin (Lam et al., 2009), showed antiproliferative effect on MCF-7 cells. French bean hemagglutinin displayed a much weaker antiproliferative activity on HepG2 cells when compared with other lectins (IC_{50} from 1 – 16 μM) (Wong and Ng, 2005c), although about 75 % of lectin devoid of antiproliferative activity on HepG2 cells.

French bean hemagglutinin potently inhibited HIV-1 reverse transcriptase with an IC_{50} of 2 μM. This activity has been shown by only some hemagglutinins with

an IC_{50} of 1 – 35 μM (Wong et al.. 2006). It may be due to protein-protein interaction as in the case of inhibition of the retroviral reverse transcriptase by the homologous protease (Böttcher and Grosse, 1997).

Lectins and hemagglutinins can be used as anti-HIV agents, anti-tumor agents and anti-fungal agents. In order to make the application practically feasible, large quantities are required. However, the yields of lectins or hemagglutinins from natural sources are low. The yield from *Phaseolus vulgaris* cultivars is usually around 10 mg from 100 g dried seeds (Sharma et al., 2009). Some may reach 100 mg level (Xia and Ng, 2006). Mass production of lectin or hemagglutinin can be achieved by the recombinant technique. The yield of recombinant *Phaseolus vulgaris* phytohemagglutinin from a 200 L fermentor is only 1.87 g (Baumgartner et al., 2002), and the cost of a fermentor and its operating expenses are very high. In this study, the yield of French bean hemagglutinin is exceedingly high, attaining gram level per 100 grams dried seeds. This extremely high yield of French bean hemagglutinin and simple purification scheme would make the applications mentioned above feasible at a very low cost.

The mechanism of antiproliferative activity of French bean hemagglutinin in MCF-7 cells was investigated. Phosphatidylserine externalization (loss of membrane asymmetry) and mitochondrial membrane depolarization are the signs of apoptosis.

French bean hemagglutinin brings about G0/G1 phase and G2/M phase arrest in MCF-7 cells. It appears that apoptosis is induced by the hemagglutinin due to the release of cytochrome c (cyt c) from the mitochondria. It has been reported that cyt c elicits G1 and G2/M cell cycle arrest and apoptosis in murine J774 cells (Hiraoka et al., 2005). In addition, from the results of annexin V-FITC/ PI staining experiment, the antiproliferative effect of the hemagglutinin on MCF-7 cells is a consequence of apoptosis.

Consistent with the loss of mitochondrial transmembrane potential, an efflux of some mitochondrial inter-membrane space proteins, like cyt c and AIF, is also considered as a critical regulatory process in mitochondrion-dependent apoptosis (Kim et al., 2006b).

When MCF-7 cells treated with 5 μM hemagglutinin, 43.1 % and 31.4 % of the cells underwent early apoptosis and late apoptosis, respectively. In the same condition, only 28.7 % cells showed mitochondrial membrane depolarization. It is due to mitochondria degradation in cells in late apoptosis and part of cells in early apoptosis. As a result, the mitochondrial membrane depolarization cannot be detected and showing a relative low percentage of cells with mitochondrial membrane depolarization (Kroemer et al., 2007)

The augmented expression level of Fas signifies that Fas ligands are the death

receptor involved in the apoptotic pathway. There is no bimane-labeled (fluorescent) hemagglutinin located intracellularly indicating that the hemagglutinin did not entered into the MCF-7 cells. The association between the hemagglutinin and Fas ligand is probably achieved by protein–protein interaction. Subsequently this causes increased expression of Fas (Wang et al., 2008), and activation of caspase-8. The caspase-8 inhibitor, z-IETD-mfk, was used to deduce the importance of FAS and caspase-8 in the pathway. The sharp decrease in the percentage of z-IETD-mfk/hemagglutinin-treated MCF-7 cells undergoing apoptosis/necrosis shows that FAS and caspase-8 are involved in the apoptotic pathway.

The Western blots demonstrate that the protein level of truncated Bid, a pro-apoptotic protein, surges abruptly as the hemagglutinin concentration is raised. This is attributed to the processing of Bid into a truncated form, tBid. Simultaneously, down-regulation of Bcl-2 (anti-apoptotic protein), together with an up-regulation of p53 and Bak (pro-apoptotic proteins), are also observed in this apoptotic incident. The expression of Bax (pro-apoptotic proteins) remains unaltered. These prompt cyt c and AIF release from mitochondria. Caspase 9 is then activated, causing the truncation of Lamin A/C, which targets cell shrinkage and membrane blebbing, finally disposing the cells to apoptosis. DNA damage causes the release of p53 from the nucleus, which further increases the total expression of p53.

p53 plays a key role in cellular proliferation, differentiation and apoptosis (Harris, 1996). Morphological alterations have been reported due to over-expression of p53 (Li et al., 2003). Morphological changes, probably also due to over-expression of p53, are observed in the hemagglutinin-treated MCF-7 cells.

Hepatocellular carcinoma is resistant to Fas-mediated apoptosis (Yan et al., 2008). It can explain why the antiproliferative effect of the hemagglutinin is much more potent towards MCF-7 cells than HepG2 cells. It further proves that apoptosis is caused by interaction of the hemagglutinin with Fas ligands.

Apoptosis in tumor cells can be brought about by (i) DNA fragmentation, (ii) chromatin condensation and /or (iii) cell shrinkage/membrane blebbing. DNA fragmentation in MCF-7 cells is indiscernible, probably because there is no caspase-3 (Janicke et al., 1998), which targets DNA fragmentation. Chromatin condensation is also caspase-3 dependent. It is likely that cell shrinkage/membrane blebbing occurs in MCF-7 cell undergoing apoptosis.

There are two main pathways of apoptosis, i.e. death receptor-mediated pathway and mitochondrial pathway. Many reports illustrate that the mitochondrial pathway is the prevailing pathway of apoptosis in MCF-7 cells (Lee and Cho, 2008, Ghoneum et al., 2008, Zhou et al., 2008, Kuo et al., 2007a, Muscella et al., 2008, Kuo et al., 2007b). By comparison, there are much fewer publications revealing that death

receptor-mediated pathway is the major apoptotic pathway in MCF-7 cells (Wang et al., 2008, Kuo et al., 2007a). Estradiol activates the mitochondrial apoptotic pathway in estrogen-independent MCF-7:5C cells. It does so by stimulating proapoptotic Bax, Bak, Bim, Noxa, Puma, and p53 protein expression and eliciting the loss of mitochondrial transmembrane potential and cytochrome *c* release (Lewis et al., 2005). However, information about the proteins that lie in the apoptotic pathway in MCF-7 cells is meager. Although both pathways are implicated in apoptosis, the death receptor cannot be identified in apoptosis induced by cycloheptapeptide (Ubol et al., 2007).

Truncated Lamin targets cell shrinkage and membrane blebbing in tumor cells. There are two accounts that Lamin B is involved in the apoptotic pathway in MCF-7 cells, bile acid derivatives (Im et al., 2001) and β-lapachone (Pink et al., 2000). In the present study, it has been observed that Lamin A/C cleavage is involved in the apoptotic pathway induced in MCF-7 cells by French bean hemagglutinin.

There are few studies about the pathway involved in lectin/ hemagglutinin-induced apoptosis cells death in the literature. Nevertheless, information on cell lines other than MCF-7 is available. Wheat germ agglutinin brings about a loss of transmembrane potential, disruption of the inner mitochondrial membrane, liberation of cytochrome c and caspase-9 activation in the Jurkat cell line.

However, the mitochondrial apoptotic events are independent of Bax, Bak and Bcl-2 (Gastman et al., 2004). Targeting of galectin-1 to glycotopes on Fas and subsequent activation of the apoptotic death-receptor pathway occur in the T-cell lines Jurkat and MOLT-4 (Brandt et al., 2008). *Viscum album coloratum* (mistletoe) agglutinin-induced apoptotic COLO cell death is attributed to the activation of caspases-2, -3, -8 and -9 and suppression of receptor interacting protein, nuclear factor-kappaB, X-linked inhibitor of apoptosis protein, and Akt/protein kinase B, partly through the tumor necrosis factor receptor 1 signaling pathway. Both death receptors pathways (activation of caspase-2, -3, -8) and mitochondrial pathway (activation of caspases-2 and -9) appear to be involved in the action of mistletoe lectin. TNFR1 induces both receptor-mediated and mitochrondria-mediated apoptosis (Khil et al., 2007). *Polygonatum cyrtonema* lectin induces an upregulation of Bax protein, a downregulation of Bcl-XL and Bcl-2 proteins, collapse of mitochondrial membrane potential, release of cytochrome c, and activation of caspases-9 and -3. Thus the lectin triggers apoptosis and autophagy via a mitochondrial-mediated ROS–p38–p53 pathway in A375 cells (Liu et al., 2008b). TNFR-mediated apoptosis is the predominant pathway induced by *Pouteria torta* lectin (pouterin) in HeLa cells, by inducing upregulation of TNFR1 and TRAF2, downregulation of TRADD, p65 NFKB subunit and IAP1, and finally nuclear fragmentation. Pouterin also induces an

overexpression of p21, which is an indicator of cell cycle arrest (Boleti et al., 2008).

We present herein the first report demonstrating hemagglutinin-induced apoptosis in MCF-7 cells and the apoptotic pathway concerned.

Chapter 5: Passiflin, a Novel Dimeric Antifungal Protein from Seeds of the Passion Fruit

5.1. Introduction

Passiflora edulis is a plant belonging to Family *Passifloraceae*. Various parts of this plant are biologically active. Its leaf extract exerts an antioxidant action (Ferreres et al., 2007) while its rind extract produces an antihypertensive effect (Ichimura et al., 2006, Tapp et al., 2008). The fruit extract displays anti-inflammatory (Vargas et al., 2007, Montanher et al., 2007), anxiolytic (Barbosa et al., 2008, Coleta et al., 2006), and antioxidant (Talcott et al., 2003) activities. In addition, the fruit extract can heal open wounds (Garros Ide et al., 2006), colonic anastomosis (Bezerra et al., 2006), gastric sutures (Silva et al., 2006), abdominal wall wound (Gomes et al., 2006) and bladder wound (Gonçalves Filho et al., 2006) in the rat model.

Glycosides (Christensen and Jaroszewski, 2001), cycloartane triterpenoids (Yoshikawa et al., 2000a, b), saponins (Yoshikawa et al., 2000a), low-methoxyl pectin (Yapo and Koffi, 2006), hydroxynitrilases (Asano et al., 2005), and antifungal proteins resembling 2S albumin in partial amino acid sequence (Agizzio et al., 2003, Pelegrini et al., 2006) have been reported from *P. edulis*. In view of the relatively scanty information on proteinaceous constituents of *P. edulis*, we undertook the present investigation to isolate an antifungal protein from the seeds of this plant.

In order to protect themselves from assault of pathogenic fungi, living organisms produce a variety of molecules including antifungal proteins. In plants, antifungal proteins have been isolated from a variety of tissues including fruits (Wang and Ng, 2002a), seeds (Wang and Ng, 2001a, b), bulbs (Wang and Ng, 2002b), rhizomes (Wang and Ng, 2005a), and roots (Lam and Ng, 2001). These antifungal proteins exhibit a wide range of molecular masses and amino acid sequences.

The antifungal protein isolated from *P. edulis* in this study, designated as passiflin, has an N-terminal sequence closely resembling that of the whey protein β-lactoglobulin. The various biochemical characteristics and biological activities of this novel antifungal protein are presented herein in comparison with other antifungal proteins and bovine β-lactoglobulin.

5.2. Materials and Methods

5.2.1. Materials

Fresh seeds (100 g) were collected from *P. edulis* (passion fruits) purchased from a local vendor. Rabbit-anti-bovine-β-lactoglobulin antiserum, bovine β-lactoglobulin, and nystatin were obtained from Sigma Chemical Company, St. Louis, Missouri, U.S.A.

5.2.2. Isolation of antifungal protein

The crude extract of *P. edulis* seeds was chromatographed on a 5 x 10 cm column of Q-Sepharose in 20 mM Tris-HCl buffer (pH 7.4). Unadsorbed proteins were eluted with the same buffer to yield fraction Q1 while adsorbed proteins were eluted stepwise, first with 0.1 M NaCl in the Tris-HCl buffer to yield fraction Q2, and then with 0.5 M NaCl added to the Tris-HCl buffer to yield fractions Q3, Q4, and Q5. Fraction Q4 in 20 mM Tris-HCl buffer (pH 7.4) containing 1.5 M ammonium sulfate was subjected to hydrophobic interaction chromatography on a 2.5 x 10 cm column of Phenyl-Sepharose. The column had been equilibrated with 20 mM Tris-HCl buffer (pH 7.4) containing 1.5 M ammonium sulfate. After unadsorbed proteins had come off the column as fraction PS1, the column was eluted with 20 mM Tris-HCl buffer (pH 7.4) to give fraction PS2. Fraction PS2 was further purified on a DEAE-cellulose column in 20 mM Tris-HCl buffer (pH 7.4).

After elution of unadsorbed proteins (D1), the column was eluted with a 0 to 1 M linear NaCl concentration gradient to yield fractions D2 and D3. Fraction D2 was subjected to final purification on a Superdex 75 10/300 GL column in 100 mM NH_4HCO_3 buffer (pH 7.4). The first peak (S1) constituted purified antifungal protein, which was designated as passiflin.

5.2.2. Protein determination was described in Section 2.2.3.

5.2.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, determination of native molecular weight by gel filtration and N-terminal amino acid sequence analysis of passiflin were described in Section 2.2.2.

5.2.4. Assay of antifungal activity was described in Section 2.2.7.

5.2.5. Assay for HIV-1 reverse transcriptase inhibitory activity was described in Section 2.2.8.

5.2.6. Assay of antiproliferative activity on tumor cell lines was described in Section 2.2.9.

5.2.7. Western blot analysis

Passiflin and β-lactoglobulin were subjected to electrophoresis, and then transferred to an immobilon-P transfer membrane (Millipore, MA, USA) using a semi-dry transfer system (Bio-Rad). The membrane was blocked with 5 % skim milk in TBS-T. The membrane was incubated with a

rabbit-anti-bovine-β-lactoglobulin polyclonal antiserum (1:500 dilution) overnight at 4 °C, followed by several washes with TBS-T. The signal was developed by incubation with anti-rabbit horseradish peroxidase-conjugated secondary antibodies (GE Healthcare) for 1.5 hour, followed by several washes with TBS-T. Enhanced chemiluminescence detection reagents (GE Healthcare) were used for detection (Lai et al., 2006).

5.3. Results

Ion exchange chromatography of *P. edulis* seed extract on Q-Sepharose produced a very large unadsorbed fraction (Q1) and two adsorbed fractions (Q2 eluted with 0.1 M NaCl, and Q3, Q4, and Q5 eluted with 0.5M NaCl). Antifungal activity resided only in fraction Q4 (Figure 5.1). This fraction was separated on Phenyl-Sepharose into an unadsorbed fraction (PS1) devoid of antifungal activity and an adsorbed fraction (PS2) with antifungal activity (Figure 5.2). Fraction PS2 was subsequently resolved on DEAE-cellulose into a large unadsorbed fraction (D1) and two smaller adsorbed fractions (D2 and D3). Antifungal activity was confined to the adsorbed fraction D2 eluted within the 0 – 0.6 M NaCl gradient (Figure 5.3). This active fraction D2 was subjected to final purification on Superdex 75 10/300 GL. Four fractions, S1 to S4, were obtained (Figure 5.4). Antifungal activity resided in the first fraction (S1). The first fraction demonstrated a single 34-kDa band in SDS-PAGE (Figure 5.5) and a single 67-kDa peak upon rechromatography on Superdex 75 10/300 GL (not shown). A summary of purification of the antifungal protein is presented in Table 5.1. The N-terminal amino acid sequence of the antifungal protein was highly homologous to bovine β-lactoglobulin, but was distinct from sequences of published antifungal proteins (Table 5.2). It inhibited mycelial growth in *R. solani* (Figure 5.6a) with an IC_{50} value of 16 ± 0.9 μM (n = 3)

(Figure 5.6b), but not in *M. arachidicola* and *F. oxysporum* when tested up to 100 μM. The antifungal protein inhibited proliferation of MCF-7 tumor cells with an IC_{50} near 15 ± 1.2 μM (n = 3) (Figure 5.7), but there was no inhibition toward HepG2 cells when tested up to 100 μM. For comparison, bovine β-lactoglobulin was tested for the various aforementioned activities and found to be lacking in these activities (Figure 5.6a and 5.7). Both the antifungal protein and bovine β-lactoglobulin were devoid of inhibitory activity on HIV-1 reverse transcriptase when tested up to 100 μM (not shown). The comparison of pharmacological activities of passiflin to various positive controls used in this study, including doxorubicin, nystatin and *B. campestris* lipid transfer protein, is summarized in Table 5.3. Western blotting of bovine β-lactoglobulin using a rabbit-anti-bovine-β-lactoglobulin antiserum yielded positive results. In contrast, there was no cross-reactivity of passiflin with the same antiserum (Figure 5.8).

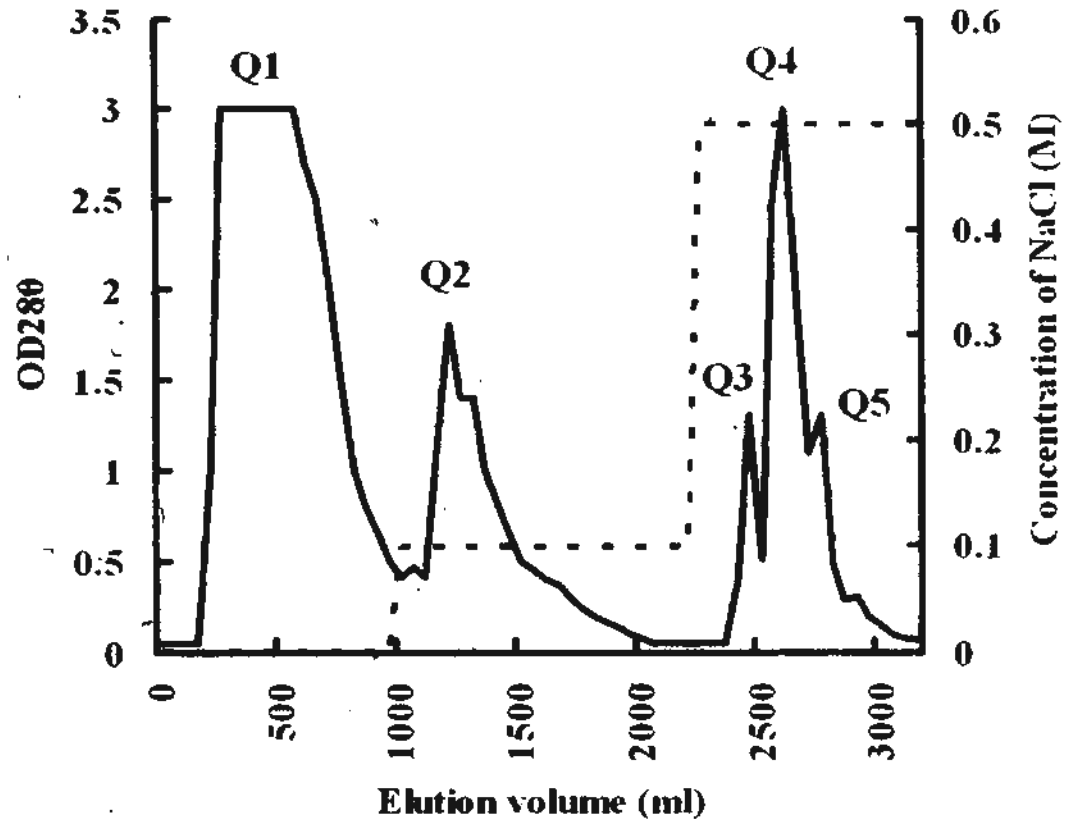


Figure 5.1: Ion exchange chromatography of *Passiflora edulis* seed extract on Q-Sepharose. The dotted lines indicate the use of 0.1 M NaCl in 20 mM Tris-HCl buffer (pH 7.4) to elute fraction Q2 and 0.5 M NaCl in 20 mM Tris-HCl buffer (pH 7.4) to elute fraction Q3, Q4, and Q5. Flow rate = 10 ml/min.

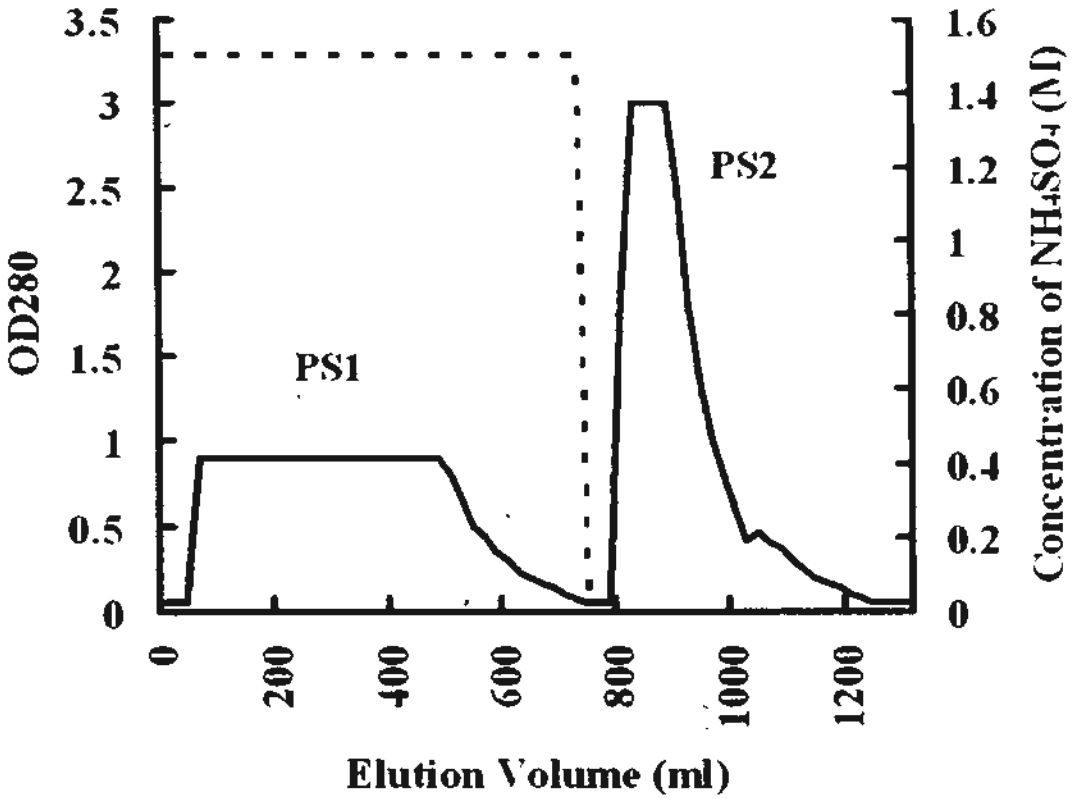


Figure 5.2. Hydrophobic interaction chromatography of fraction Q4 on Phenyl-Sepharose. The dotted line indicates the use of 1.5 M $(NH_4)_2SO_4$ in 20 mM Tris-HCl buffer (pH 7.4) to elute fraction PS1. Twenty millimolar Tris-HCl buffer (pH 7.4) was used to elute fraction PS2. Flow rate = 5 ml/min.

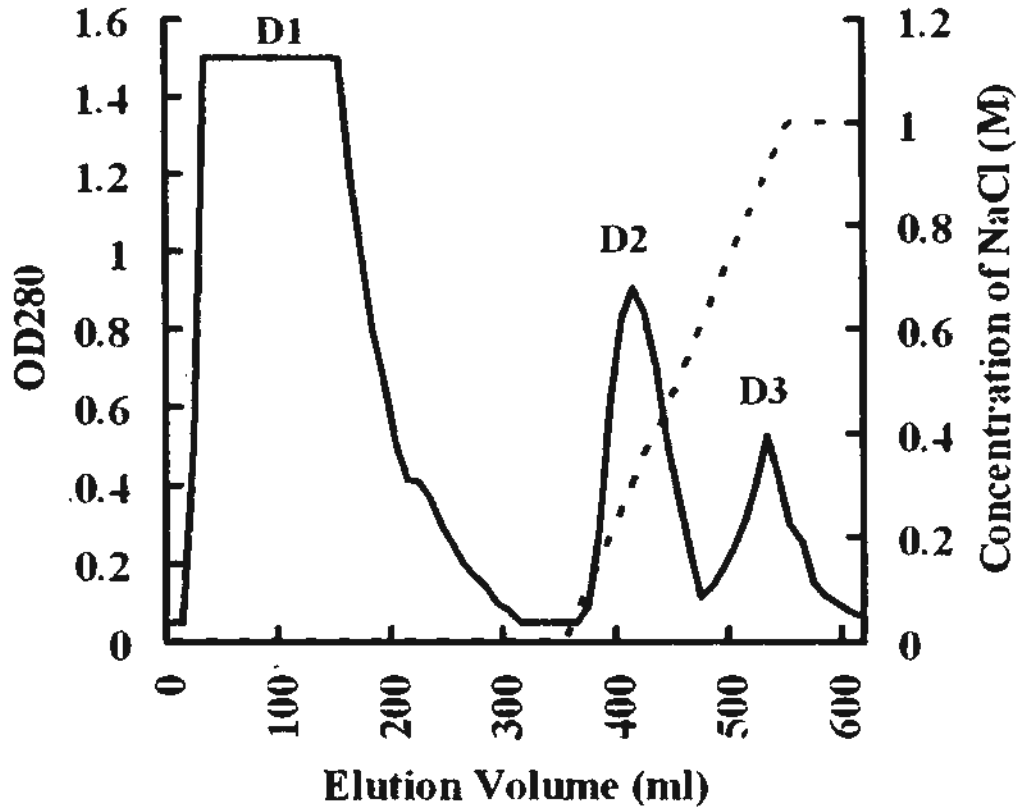


Figure 5.3. Ion exchange chromatography of fraction PS2 on DEAE-cellulose. The dotted line indicates the use of a linear 0 – 1 M NaCl gradient to elute fractions D2 and D3. Flow rate = 5 ml/min.

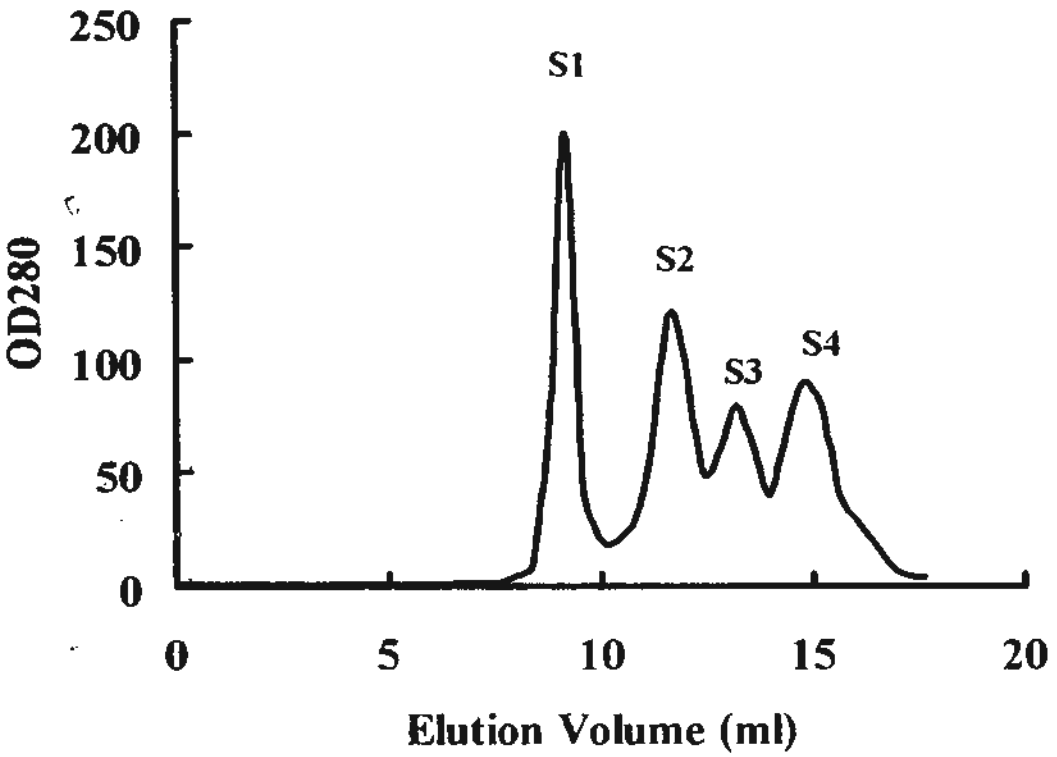


Figure 5.4. Gel filtration of fraction D2 on Superdex 75 10/300 GL. Flow rate = 0.5 ml/min. Antifungal activity was located only in fraction S1 with purified antifungal protein designated as passiflin.

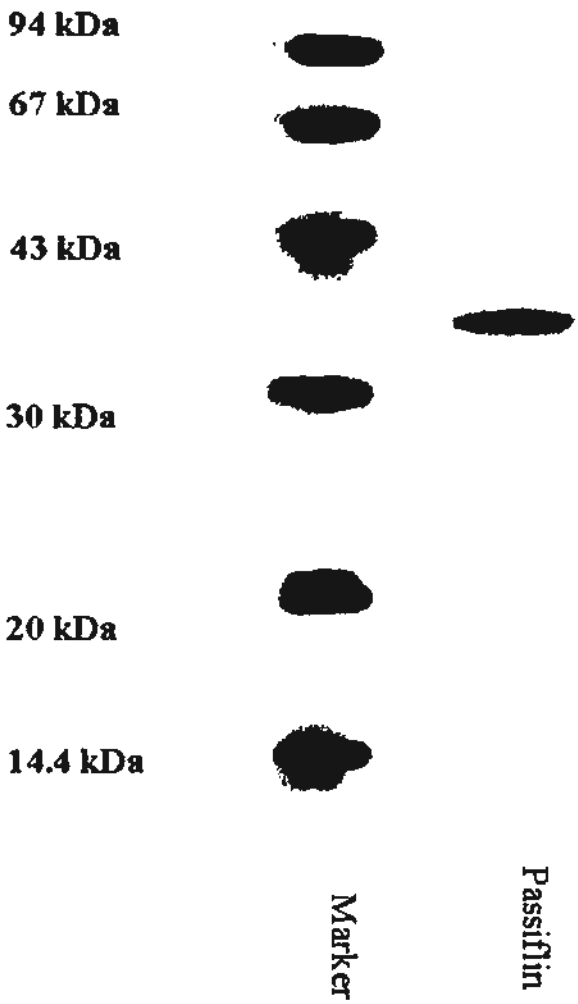


Figure 5.5. SDS-polyacrylamide gel electrophoresis. Right lane: purified passiflin. Left lane: Molecular mass markers from GE Healthcare including phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14.4 kDa).

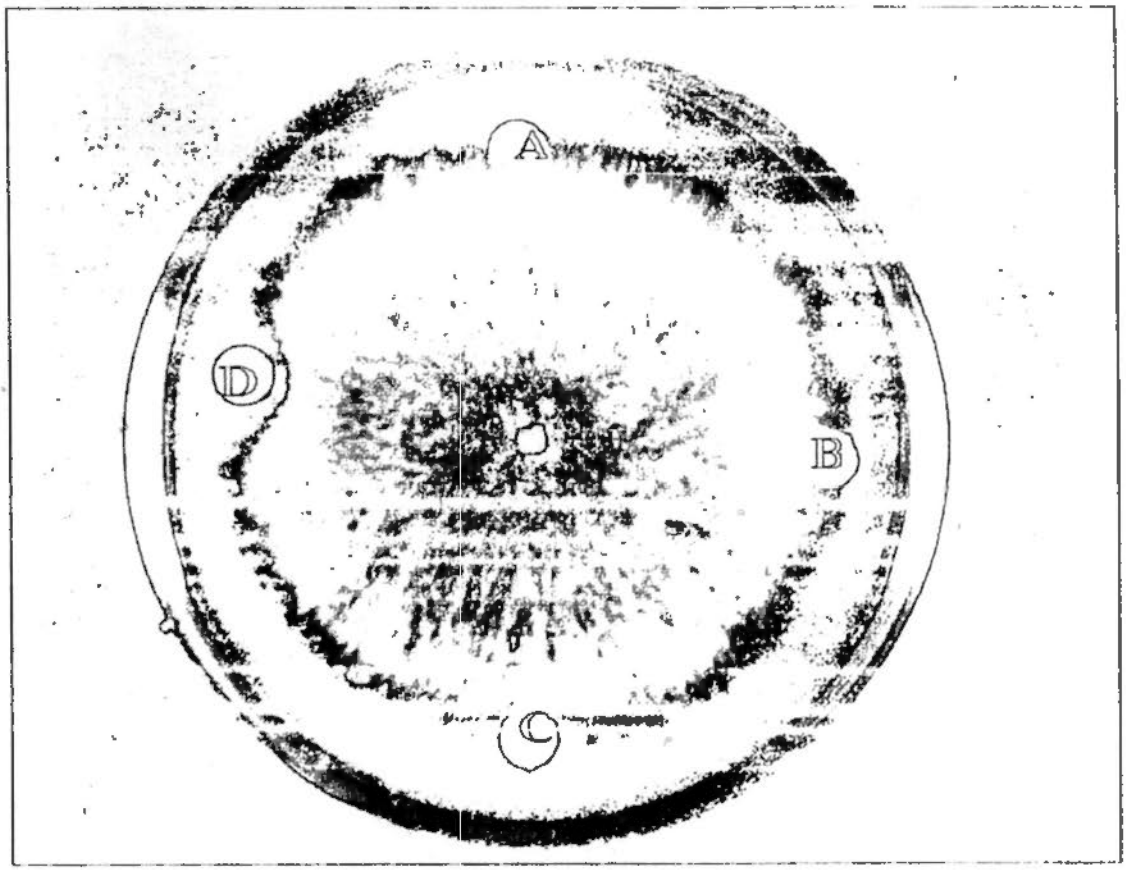


Figure 5.6 (a) Test of passiflin and bovine β-lactoglobulin for antifungal activity toward *Rhizotonia solani*. A: buffer control. B: 30 μmol (1 mg) bovine β-lactoglobulin. C: 30 μmol (2 mg) passiflin. D: 10 units nystatin (positive control).

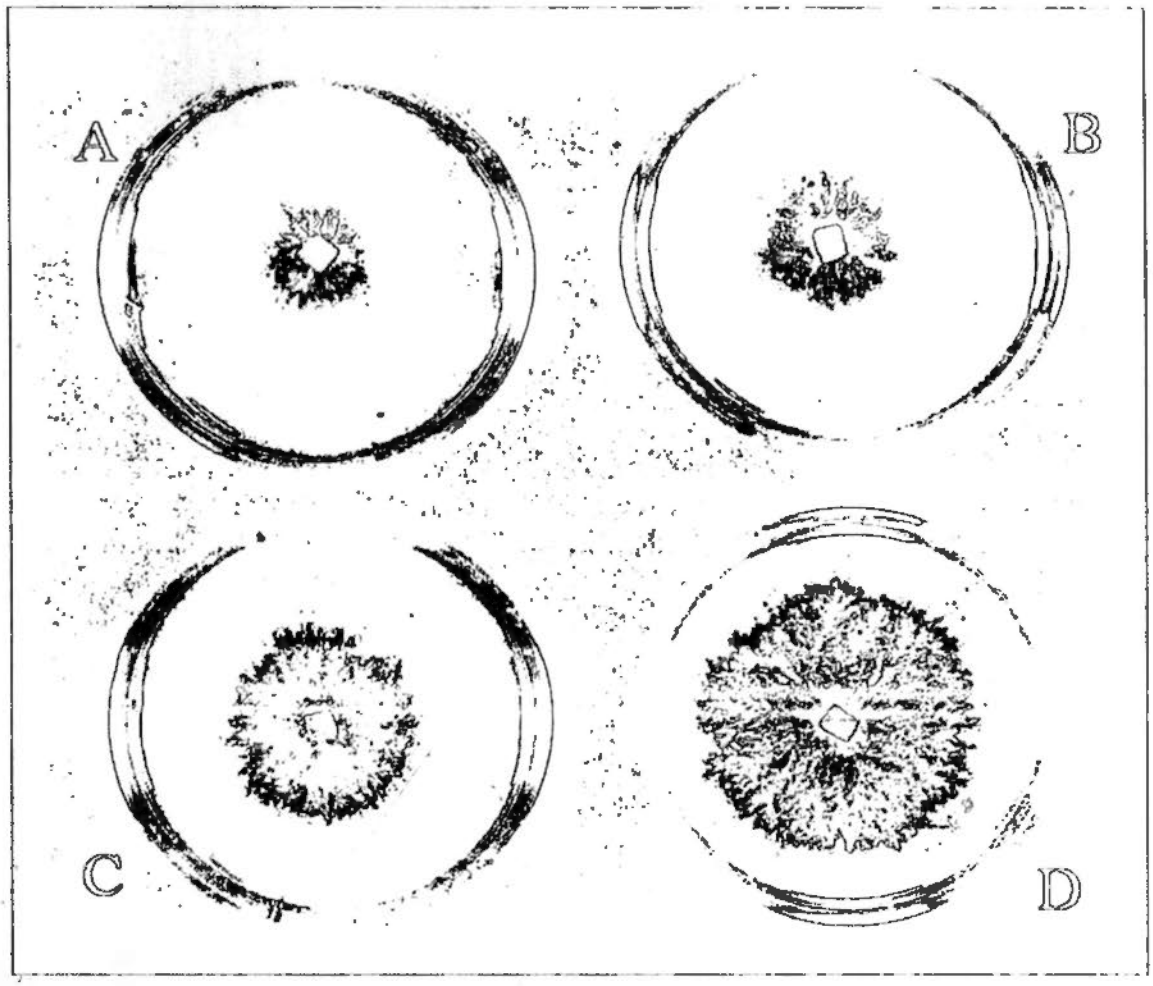


Figure 5.6 (b) Determination of IC_{50} antifungal activity of passiflin toward *Rhizotonia solani*. A: 100 μM passiflin, B: 40 μM passiflin. C: 15 μM passiflin. D: buffer control. IC_{50} was found to be 16 ± 0.9 μM based on 3 experiments. Figure represented one of three experiments.

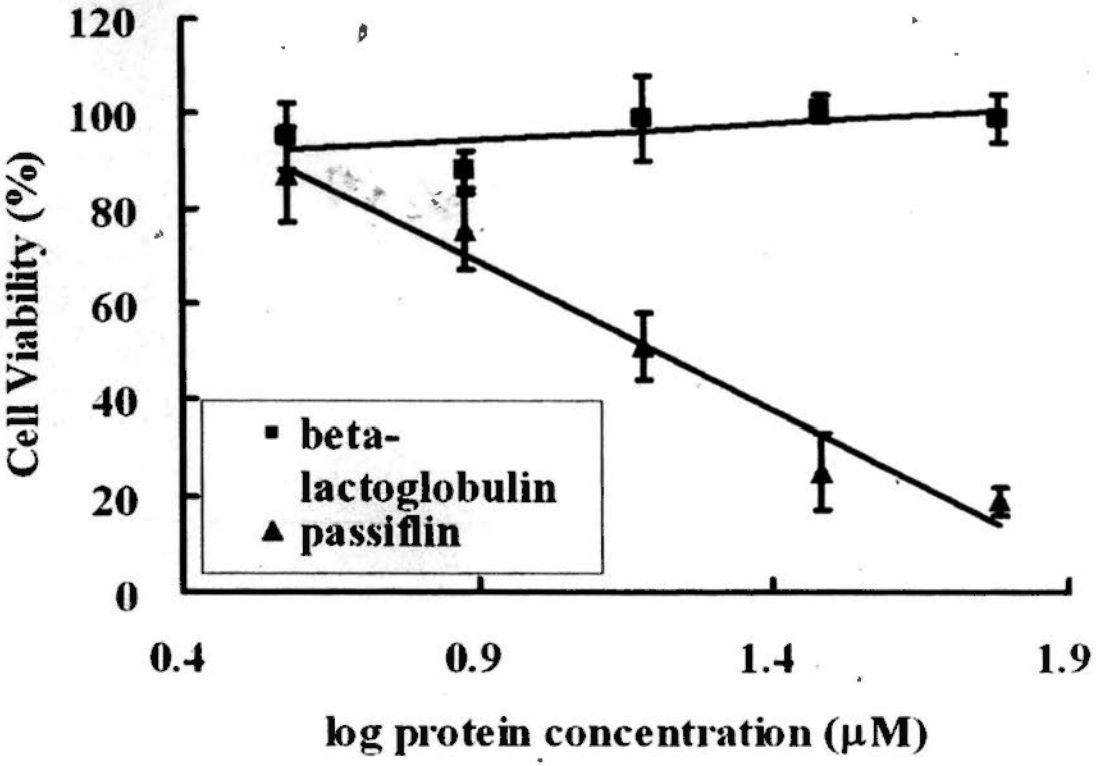


Figure 5.7. Effects of passiflin and bovine β-lactoglobulin on viability of breast cancer MCF-7 cells after incubation for 48 hous. IC_{50} of passiflin on viability of MCF-7 cells was found to be 15 ± 1.2 μM based on 3 experiments. Values are expressed as mean ± SD (N=3).

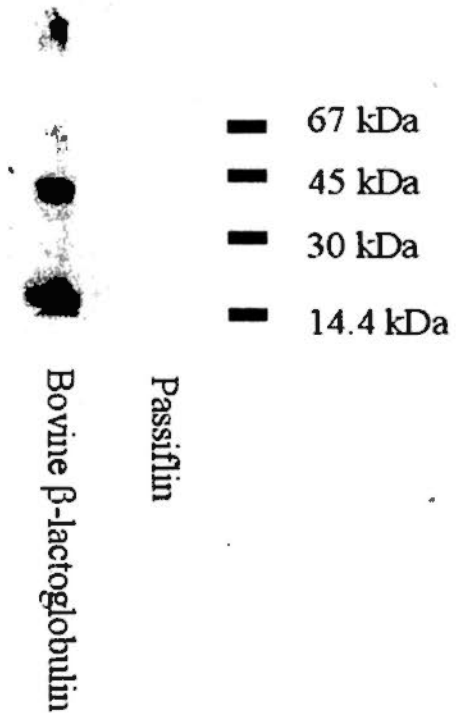


Figure 5.8. Western blot of bovine β-lactoglobulin and passiflin. Left lane: bovine β-lactoglobulin. Cross-reactivity of both monomer (18.3 kDa) and dimer (36.6 kDa) with rabbit-anti-bovine-β-lactoglobulin antiserum. Right lane: purified passiflin. There was no cross-reactivity of passiflin with rabbit-anti-bovine-β-lactoglobulin antiserum. Molecular weights were determined using molecular weight markers.

Table 5.1. Protein yields of antifungal protein from 100 g fresh *Passiflora edulis* seeds at different stages of purification.

Column	Chromatographic fraction	Yield (mg)
-	Crude Extract	2050
Q-Sepharose	Q4	830
Phenyl-Sepharose	PS2	340
DEAE-cellulose	D2	32
Superdex 75	S1 (passiflin)	5

Table 5.2. Comparison of N-terminal amino acid sequence of passiflin with mammalian β-lactoglobulins and previously isolated antifungal peptide and protein from passion fruit.

	Residue No.	Sequence
Passiflin	1	AFLDIQKVAGTWYSLA
Bovine β-lactoglobulin	8	KGLDIQKVAGTWYSLA
Bubalus bubalis β-lactoglobulin	8	KGLDIQKVAGTWYSLA
Capra hircus β-lactoglobulin	8	KGLDIQKVAGTWYSLA
Rangifer tarandus β-lactoglobulin	8	KDLDVQKVAGTWYSLA
Mouflon β-lactoglobulin	8	KGLDIQKVAGTWYHLA

2S albumin-like antifungal peptide (Agizzio et al., 2003)	1	QSERFEQQMQGQDFSHDERFL SQAA
2S albumin-like antifungal protein (Pelegrini et al., 2006)	1	PSERCRRQMQGDFS

Identical amino acid residues are underscored.

Table 5.3. Comparison of biological potencies of passiflin, doxorubicin, nystatin and *Brassica campestris* lipid transfer protein.

	Passiflin (IC_{50} in μM)	Doxorubicin (IC_{50} in μM)	Nystatin (IC_{50} in μM)	*B. campestris* lipid transfer protein (IC_{50} in μM)
Antiproliferative activity against HepG2 cells	-	11.5 ± 2.1	-	6.2 ± 5.4
Antiproliferative activity against MCF-7 cells	15 ± 1.2	4.3 ± 1.6	-	38 ± 3.6
Antifungal activity against *Rhizoctonia solani*	16 ± 0.9	-	10.2 ± 2.7	-
Inhibitory activity against HIV-1 reverse transcriptase	-	-	-	4.6 ± 1.2

5.4. Discussion

The antifungal protein purified in the present study is unique in its possession of an N-terminal amino acid sequence with remarkable homology to bovine β-lactoglobulin. Antifungal proteins with such a structure have not been encountered before. It is extremely interesting since passiflin is of plant origin while β-lactoglobulin is a mammalian whey protein. Despite this structural resemblance, there are many points of dissimilarities between the two proteins indicating that they are distinct proteins. The molecular mass of passiflin (67 kDa) is higher than that of β-lactoglobulin (36.6 kDa). In addition, intact β-lactoglobulin does not demonstrate antifungal or antiproliferative activity whereas passiflin is endowed with these activities, indicating differences in biological activities between the two proteins. However, β-lactoglobulin hydrolysate manifested antifungal activity (Hernández-Ledesma et al., 2008). Furthermore, passiflin shows no cross-reactivity with a rabbit-anti-bovine-β-lactoglobulin antiserum suggesting immunological distinctiveness between them.

Passiflin is devoid of inhibitory activity toward HIV-1 reverse transcriptase is also noteworthy since some plant proteins comprising protease inhibitors (Ye et al., 2001a), lectins (Wong and Ng, 2005b) and antifungal proteins (Wong and Ng, 2005c) display this antiretroviral activity. β-lactoglobulin lacks HIV-1 reverse transcriptase

inhibitory activities.

The antifungal activity of passiflin is species-specific. It impedes mycelial growth in *R. solani,* but not in other fungi such as *F. oxysporum* and *M. arachidicola.* This observation is reminiscent of similar findings in case of antifungal proteins from asparagus seeds (Wang and Ng, 2001b) and shallot bulbs (Wang and Ng, 2002b). These two antifungal proteins inhibit only one out of the several fungal species tested.

Passiflin manifests a potent inhibitory action on breast cancer cells with an IC_{50} value of 15 μM. This finding is in keeping with previous demonstrations of the antiproliferative action of some antifungal proteins including ribosome-inactivating proteins (Tsao et al., 1990) and defensins (Wong and Ng, 2005c). Interestingly, passiflin has no inhibitory activity toward hepatoma HepG2 cells, illustrating a specificity of action. Likewise, the ribosome-inactivating proteins trichosanthin and momorcharin exert highly potent inhibitory activity against choricarcinoma but are much less active toward hepatoma cells (Tsao et al., 1990).

Another distinctive feature of passiflin is its chromatographic behavior on Q-Sepharose and DEAE-cellulose. Most of the antifungal proteins are unadsorbed on these anion exchangers whereas passiflin is adsorbed. The chromatographic procedure employed for purification of passiflin is highly efficient since it removes a

considerable amount of materials without antifungal activity at each step.

Passiflin is distinct from the 2S albumin-like antifungal protein and peptide previously isolated from seeds of passion fruit (Pelegrini et al., 2006, Agizzio et al., 2003), as evidenced by differences in molecular mass, N-terminal amino acid sequence, and species-specificity of antifungal activity. Passiflin is devoid of antifungal activity toward *F. oxysporum* which, however, is susceptible to the 2S albumin-like antifungal protein and peptide.

In summary, passiflin isolated from *P. edulis* is a distinctive dimeric antifungal protein. To date, only a small number of antifungal proteins have been shown to be dimeric, e.g. those from sanchi ginseng (Lam and Ng, 2001), Chinese ginseng (Ng and Wang, 2001), and American ginseng (Wang and Ng, 2000). Passiflin possesses a β-lactoglobulin-like N-terminal sequence. However, it does not cross-react with an anti-β-lactoglobulin antiserum. It exhibits antiproliferative and antifungal activities, which are missing in β-lactoglobulin. Thus, passiflin is biologically and immunologically unrelated to β-lactoglobulin. In this context, it is worth mentioning that thaumatin-like proteins have antifungal activity but no sweet taste while the converse is true of thaumatin although they are highly homologous in structure (Ye et al., 1999).

Chapter 6: First Report of an Antifungal Protein from *Peltophorum pterocarpum*

6.1. Introduction

Peltophorum pterocarpum is a plant belonging to the family *Caesalpiniaceae*. A paste of its stem bark prepared in water is applied topically to treat wounds and skin diseases. *P. pterocarpum* bark is commonly used in Thailand to cure gastrointestinal infections. Various parts of this plant have been shown to have biological activity. Ethanolic extracts of its bark have potent bacteriostatic and bactericidal activities with the most effective extracts active against the two strains of *E. coli* O157:H7 (Voravuthikunchai et al., 2004, Voravuthikunchai et al., 2006). Methanolic extracts of its flowers exhibit antimicrobial activity against a number of bacteria, including *Bacillus subtilis* and *Enterococcus faecalis* etc (Duraipandiyan et al., 2006).

Amidases can break down carboxylic acid amides to free carboxylic acids and ammonia. They have been purified from many bacteria, e.g. *Geobacillus pallidus* (Makhongela et al., 2007). They can also be found in plants, e.g. soy bean (Kimura et al., 1998), *Raphanus sativus* (Berger et al., 1995), *Silene alba* cells (Lhernould et al., 1995), orange flavedo (Kammermeier-Steinke et al., 1993) and in animal tissues, e.g. human serum (Hoijer et al., 1996), *Ophiophagus Hannah* (Zhang et al., 1994), porcine liver (Heymann et al., 1993), rat brain (Cooper et al., 1993), human kidney (Ishikawa et al., 1993).

Antifungal proteins are a family of proteins deployed to combat fungi pathogenic to plants and animals. They have been reported from a multitude of plants (Wang and Ng, 2005c), animals (Clément et al., 2008), bacteria (Yadav et al., 2007), and fungi (Wang and Ng 2006). Plant tissues that produce antifungal proteins and peptides comprise bulbs (Shenoy et al., 2006), rhizomes (Lam and Ng, 2001b), tubers (Flores et al., 2002), fruits (Ho and Ng, 2007), seeds (Lam et al., 2009), and roots (Wang and Ng, 2000). Plant antifungal proteins are divided, based on their structure or activity, into various types. The different types include chitinases and chitinase-like proteins (Lam and Ng, 2001c), lipid transfer proteins (Lin et al., 2007), protease inhibitors (Ng et al., 2003), ribosome inactivating proteins (Wong et al,. 2008), glucanases (Allardyc and Linton, 2008), thaumatin-like proteins (Chua et al., 2007), and defensin-like peptides (Leung et al., 2008). In view of the dearth of knowledge on proteinaceous components of *P. pterocarpum*, an antifungal amidase was purified from *P. pterocarpum* seed in the present investigation.

6.2. Material and Methods

6.2.1. Purification of antifungal amidase

Seeds (180 g) collected from *Peltophorum pterocarpum* trees were extracted by homogenization in distilled water (10 ml/g seeds). Following centrifugation of the homogenate at 20000 g for 30 min at 4 °C, Tris-HCl buffer (2 M, pH 7.4) was added to the supernatant until the final concentration of Tris reached 20 mM. The supernatant was then applied on a 5 cm x 10 cm column of Q-Sepharose (GE Healthcare). Unadsorbed proteins were eluted with 20 mM Tris-HCl buffer (pH 7.4). Adsorbed proteins were eluted stepwise, first with 100 mM NaCl, then with 500 mM NaCl, and finally with 1000 mM NaCl added to the 20 mM Tris-HCl buffer. The fraction eluted with 500 mM NaCl was taken, dialyzed, and then subjected to chromatography on a 5 cm x 10 cm column of DEAE-cellulose (Sigma). After removal of unadsorbed proteins with 20 mM Tris-HCl buffer (pH 7.4), adsorbed proteins were eluted with 100 mM NaCl, and then with 1000 mM NaCl added to the 20 mM Tris-HCl buffer. The fraction eluted with 100 mM NaCl was saved, dialyzed, and lyophilized before gel filtration chromatography on a Superdex 75 10/300 GL (GE Healthcare) column using an AKTA Purifier (GE Healthcare). The column had been calibrated with molecular mass markers, including Blue Dextran 2000 (to indicate void volume), bovine serum albumin (67 kDa), ovalbumin (43 kDa),

chymotrypsinogen A (25 kDa), myoglobulin (17.6 kDa), ribonuclease A (13.7 kDa), and aprotinin (6.5 kDa) (GE Healthcare), to determine the molecular mass of the protein. The first fraction collected represented purified antifungal amidase, which was designated as peltopterin.

6.2.2. Molecular mass determination using sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and N-terminal amino acid sequencing were described in Section 2.2.2.

6.2.3. Assay of amidase activity

Peltopterin was assayed for this activity in view of its N-terminal sequence homology to amidases. Amidase activity was determined by the release of ammonia from the hydrolysis of amides using the phenol-hypochlorite ammonia detection method (Weatherburn, 1967). The reaction mixture (300 µl) containing 25 mM iodoacetamide (as substrate), 10 µl peltopterin (0.5 mg/ml) in 50 mM Tris-HCl buffer pH 7 was incubated at 50 °C for 15 minutes. The reaction was terminated by adding to 350 µl reagent A (0.59 M phenol and 1 mM sodium nitroprusside), followed by the addition of 350 µl reagent B (2.0 M sodium hydroxide and 0.11 M sodium hypochlorite). Activity was measured spectrophotometically at 600 nm after 5 min incubation at room temperature. Standards were prepared using ammonium

chloride. One unit of enzyme activity was described as 1 mg of enzyme that catalyzed the release of 1 μmol of NH_3 per minute. Amidase activity was also tested using urea (GE Healthcare) and acrylamide (GE Healthcare) as substrates. Control reactions were carried out in the buffer only.

6.2.4. Optimum temperature and pH of amidase activity

Amidase assay using iodoacetamide (as substrate) was performed at various temperatures (10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 °C) in 50 mM Tris-HCl (pH 9) or in buffers of various pH values (pH 0 – pH 14) at 50 °C for 15 min.

6.2.5. Thermostability study of amidase

Peltopterin was incubated with iodoacetamide at various temperatures (4, 25, 30, 40, 50, 60, 70, 80, 90, and 100 °C) for 15 min. The amidase assay was then carried out.

6.2.6. Assay of antifungal activity was described in Section 2.2.6.

6.2.7. Effects of temperature and pH on antifungal activity

Peltopterin (10 μM) was incubated at various temperatures (4, 25, 30, 40, 50, 60, 70, 80, 90, and 100 °C) or in buffers of various pH values (pH 0 - pH 14) for 15

minutes. It was then cooled down to room temperature or neutralized to pH 7, respectively, immediately before assay of antifungal activity.

6.2.8. Assay of chitin deposition at hyphal tips

This assay was conducted as described in Moreno et al, (2006) to observe chitin deposition at hyphal tips of *Rhizoctonia solani*. Following incubation of *R. solani* with peltopterin for 4 hours, Congo red was added until a final concentration of 1 mM was reached. Fluorescence was examined 2 hours later by confocal microscopy using an excitation wavelength of 543 nm and an emission wavelength of 560 – 635 nm. The tips of growing hyphae would not stain with Congo red while hyphal tips with inhibited growth would be stained (Moreno et al., 2006).

6.2.9. Assay of HIV-1 reverse transcriptase inhibitory activity was described in Section 2.2.7.

6.2.10. Assay of antiproliferative activity was described in Section 2.2.8.

6.3. Results

Ion exchange chromatography of the *P. pterocarpum* seed extract on Q-Sepharose produced a large unadsorbed fraction (Q1) and three adsorbed fractions (Q2 eluted with 100 mM NaCl, a slightly larger Q3 eluted with 500 mM NaCl, and the smallest Q4 eluted with 1000 mM NaCl) (Figure 6.1). Antifungal activity resided in fraction Q3. This fraction was separated on DEAE-cellulose into a tiny unadsorbed fraction (D1) devoid of antifungal activity and two adsorbed fractions of similar size (D2 eluted with 100 mM NaCl, and D3 eluted with 1000 mM NaCl) (Figure 6.2), D2, the only fraction with antifungal activity, was subsequently resolved on Superdex 75 10/300 GL. Two fractions, S1 and S2, were obtained (Figure 6.3). Antifungal activity resided in the first fraction (S1), which appeared to be much smaller than the second fraction (S2). Fraction S1, to which antifungal activity was confined, was re-chromatographed on Superdex 75 10/300 GL. It was eluted as a single homogeneous peak with the same elution volume as before corresponding to a molecular mass of 60 kDa (not shown). The purified antifungal amidase was designated as peltopterin. It demonstrated a single 60-kDa band in SDS-PAGE (Figure 6.4). A summary of the purification of the peltopterin is included in Table 6.1. The N-terminal sequence of peltopterin was homologous to some amidases (Table 6.2).

The optimum pH and temperature of peltopterin in the amidase assay towards iodoacetamide were pH 9 and 50 °C, respectively (Figure 6.5). The amidase activity of peltopterin was reduced to 50 % and 10 % after exposure to 70 °C and 100 °C for 15 minutes (not shown). Its amidase activities toward ioacetamide, urea and acrylamide, in pH 9 and 50 °C, were 1038 ± 62, 1023 ± 82 and 998 ± 72 U/mg amidase (mean ± SD, n = 3), respectively.

Peltopterin inhibited strong mycelial growth in *R. solani*, with an IC_{50} of 0.65 ± 0.05 μM (Figure 6.6) but not in *M. arachidicola, H. maydis, F. oxysporum,* and *V. mali* (not shown). The antifungal activity of peltopterin was stable throughout the pH range 0 – 14 (Figure 6.7A). It was stable in the temperature range 0 – 100 °C (Figure 6.7B). The hyphal growth of *R. solani* was impeded, as evidenced by accumulation of chitin deposits stainable with Congo red at the hyphal tip (Figure 6.8). It inhibited HIV-1 reverse transcriptase with an IC_{50} of 27 nM (Figure 6.9). It cannot inhibit proliferation of HepG2 and MCF-7 tumor cells up to 50 μM.

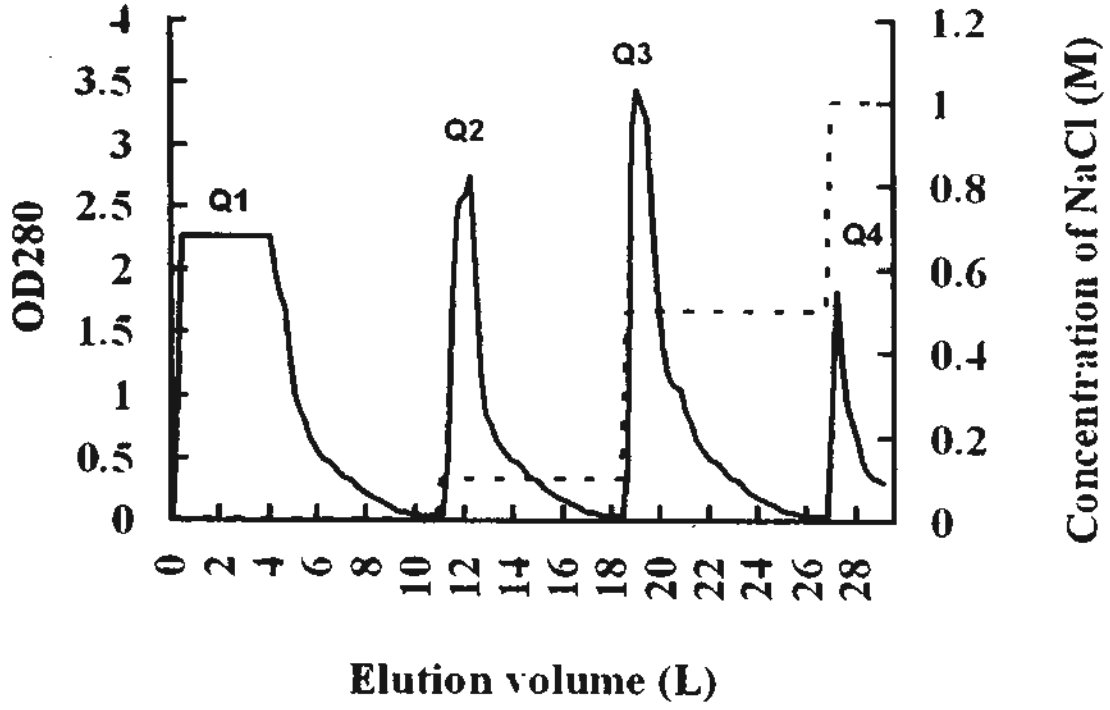


Figure 6.1. Ion exchange chromatography of *Peltophorum pterocarpum* extract on Q-Sepharose. The dotted lines indicate the use of 0.1, 0.5 and 1 M NaCl to elute fractions Q2, Q3, and Q4, respectively.

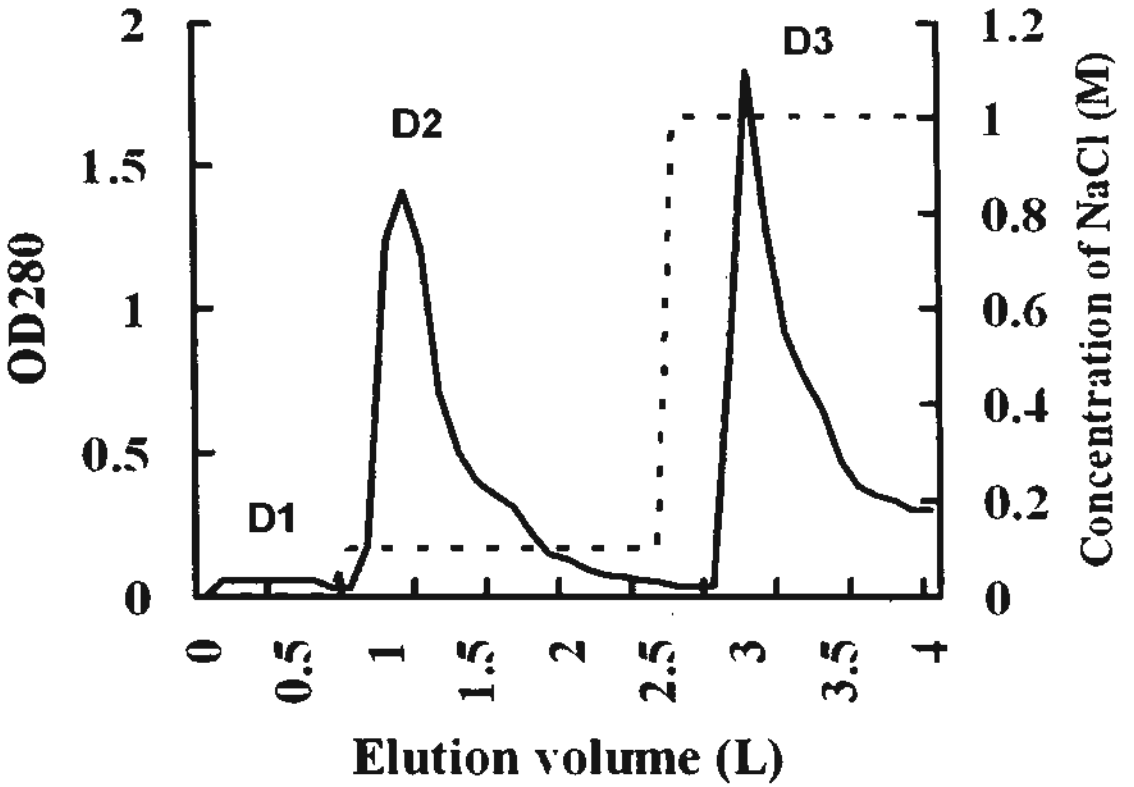


Figure 6.2. Ion exchange chromatography of Q3 fraction on DEAE-cellulose. The dotted lines indicate the use of 100 mM and 1000 mM NaCl to elute fraction D2, and D3, respectively.

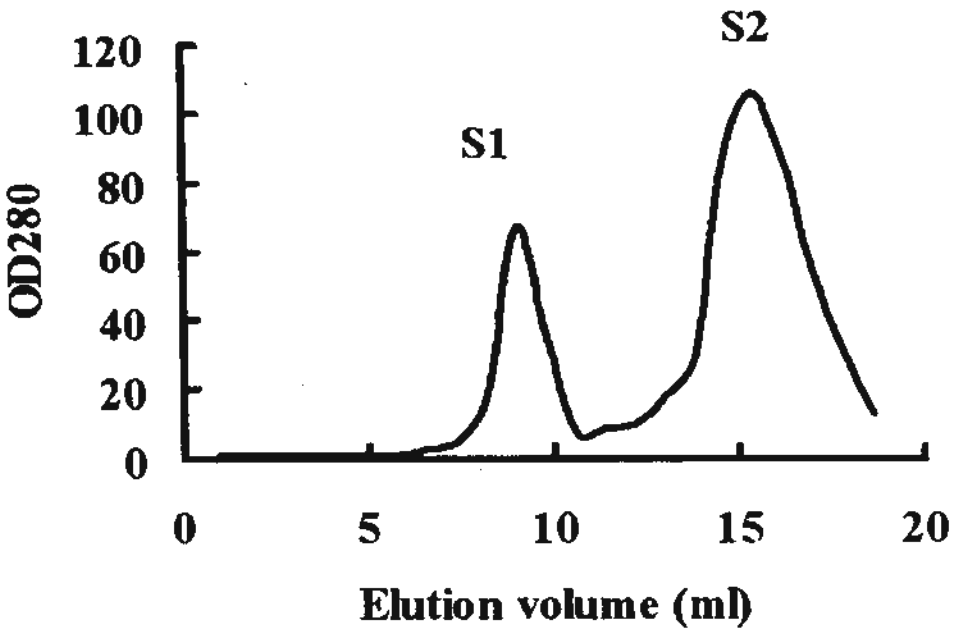


Figure 6.3. Gel filtration of fraction D2 on Superdex 75 10/300 GL. S1 represents purified antifungal amidase, which is designated as peltopterin. Flow rate = 0.5 ml/min.

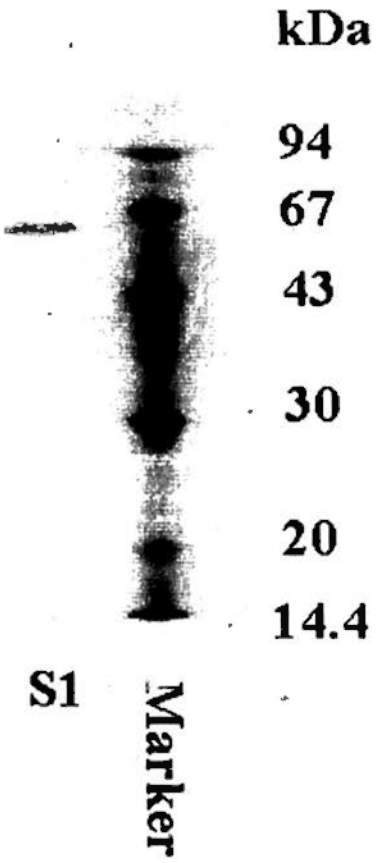
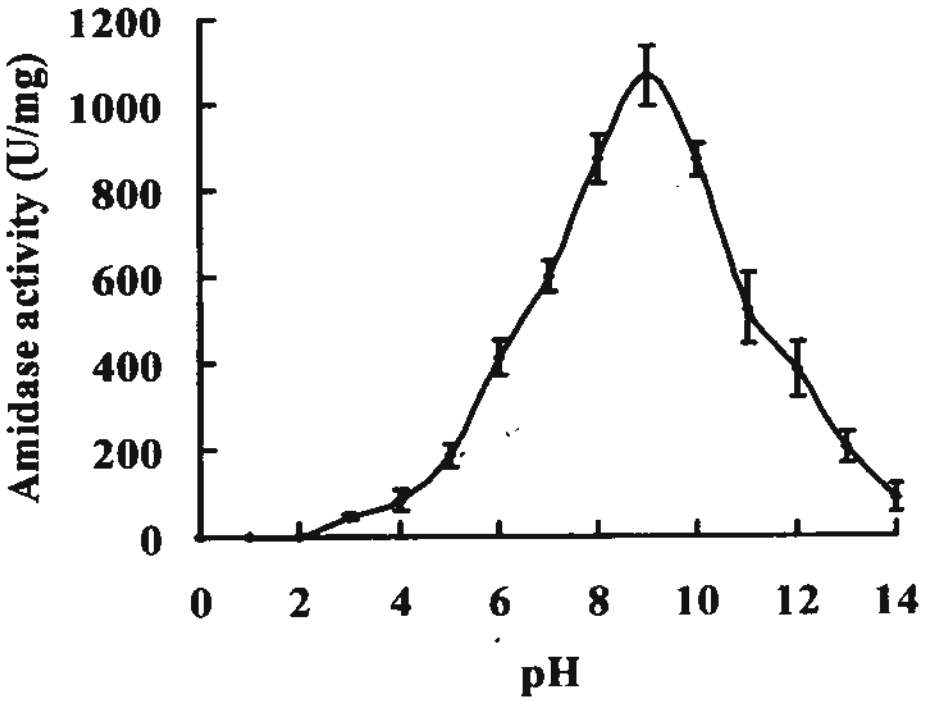


Figure 6.4. SDS-polyacrylamide gel electrophoresis. Left lane: Fraction S1 representing peltopterin. Right lane: Molecular mass markers from GE Healthcare including phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa) and soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14.4 kDa).

(5A)



(5B)

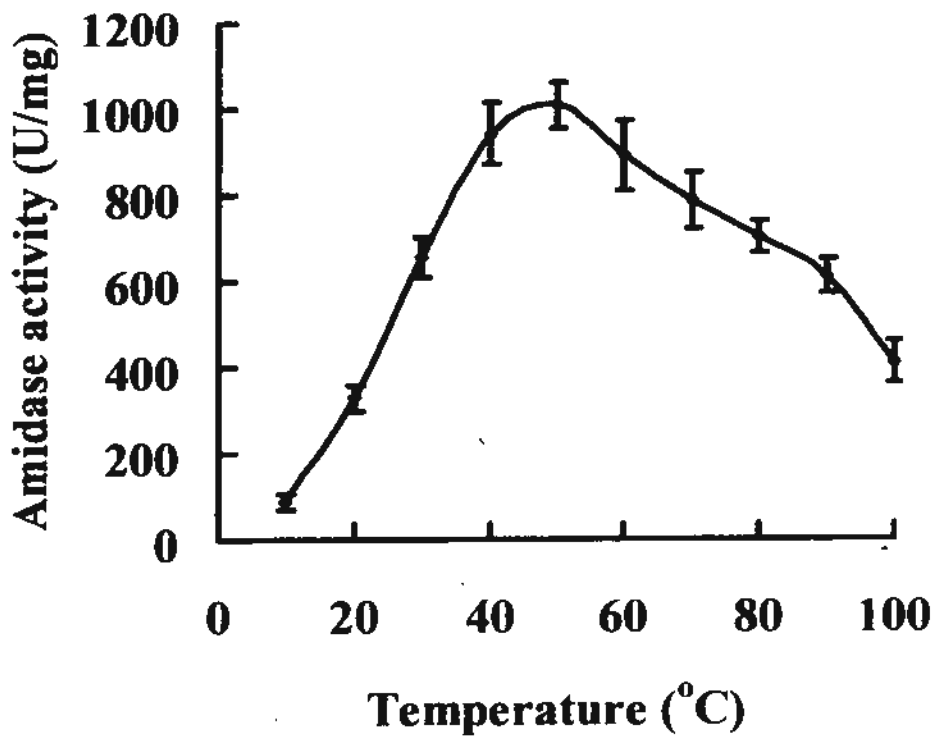


Figure 6.5. Amidase activity of peltopterin at (5A) various pH values and (5B) temperatures.

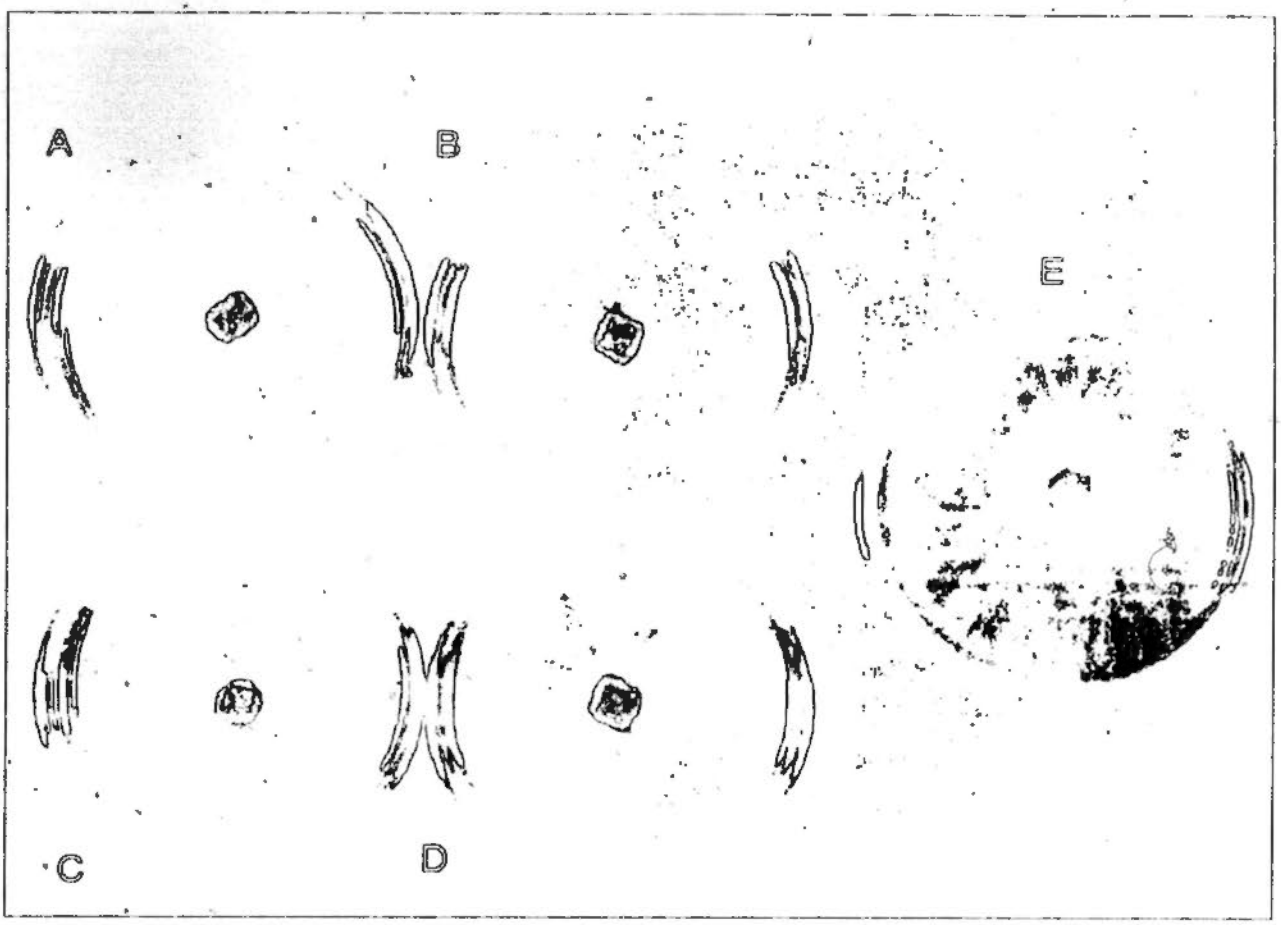
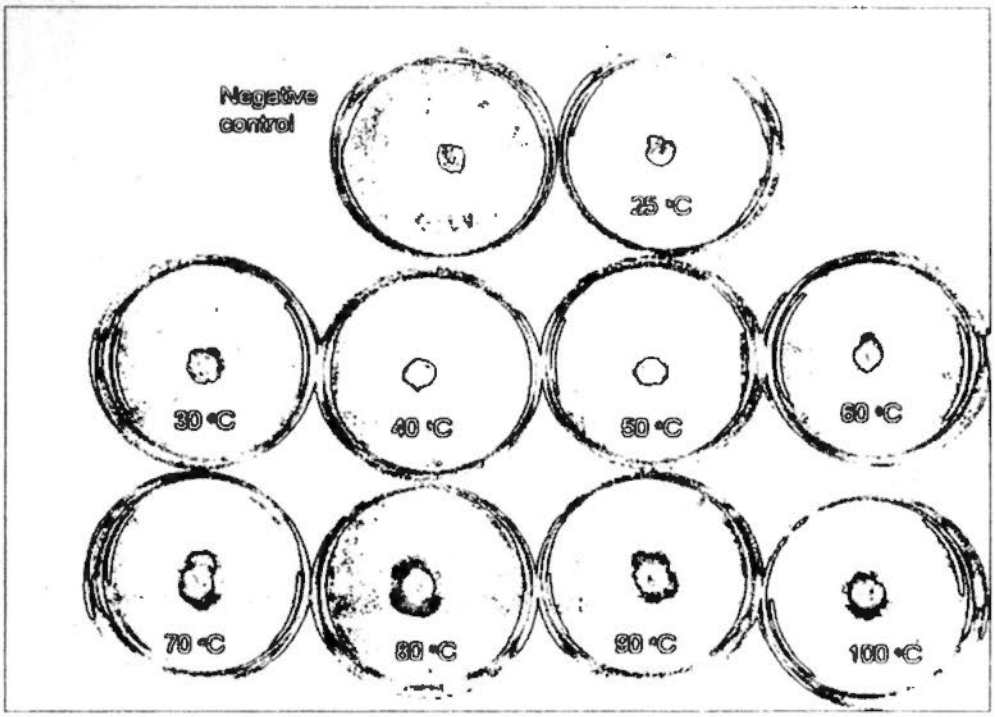


Figure 6.6. Antifungal activity of peltopterin toward *Rhizoctonia solani*. Figure 3A: 10 μM peltopterin, B: 2.5 μM peltopterin. C: 0.625 μM peltopterin. D: 0.156 μM peltopterin. E: buffer control.

(7A)



(7B)

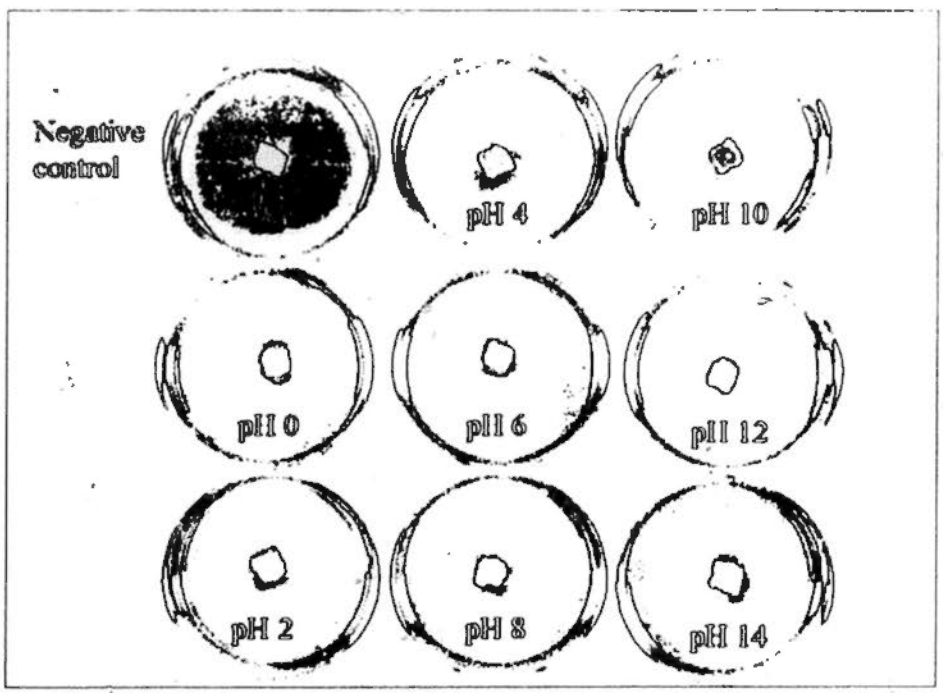


Figure 6.7. Antifungal activity of peltopterin toward *Rhizoctonia solani* after different temperatures and pH treatment. (7A) 10 μM peltopterin was incubated at various temperatures (25 – 100 °C). (7B) 10 μM peltopterin was incubated at different pH values of buffers. The assay was performed after neutralized to pH 7.

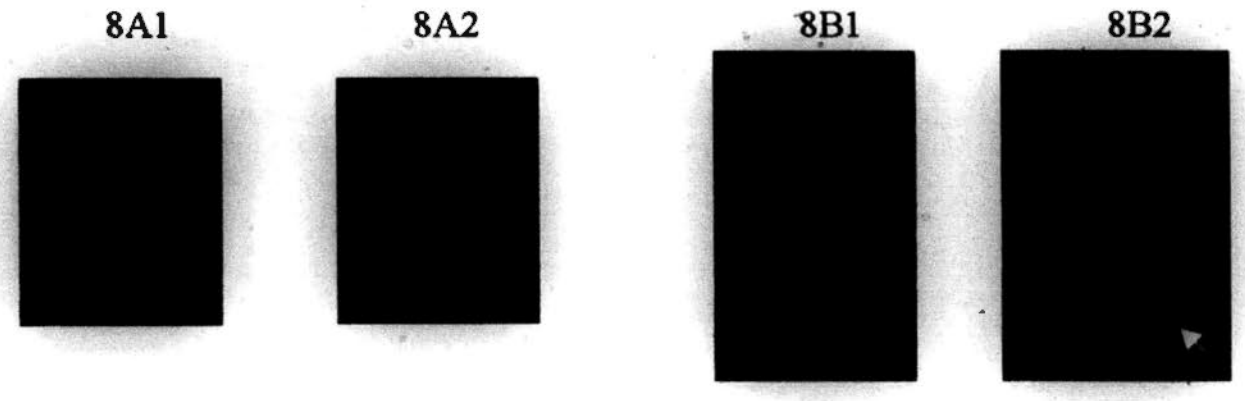


Figure 6.8. Congo red staining showing chitin deposition at the hyphal tips of *R. solani*.

(8A) Buffer control. 8A1 and 8A2 pictures were taken under the light microscope and fluorescence microscope respectively.

(8B) Treatment with 10 μM peltopterin. 8B1 and 8B2 pictures were taken under the light microscope and fluorescence microscope, respectively. The hyphal tips were stained with Congo red (white arrow) showing that the growth was inhibited.

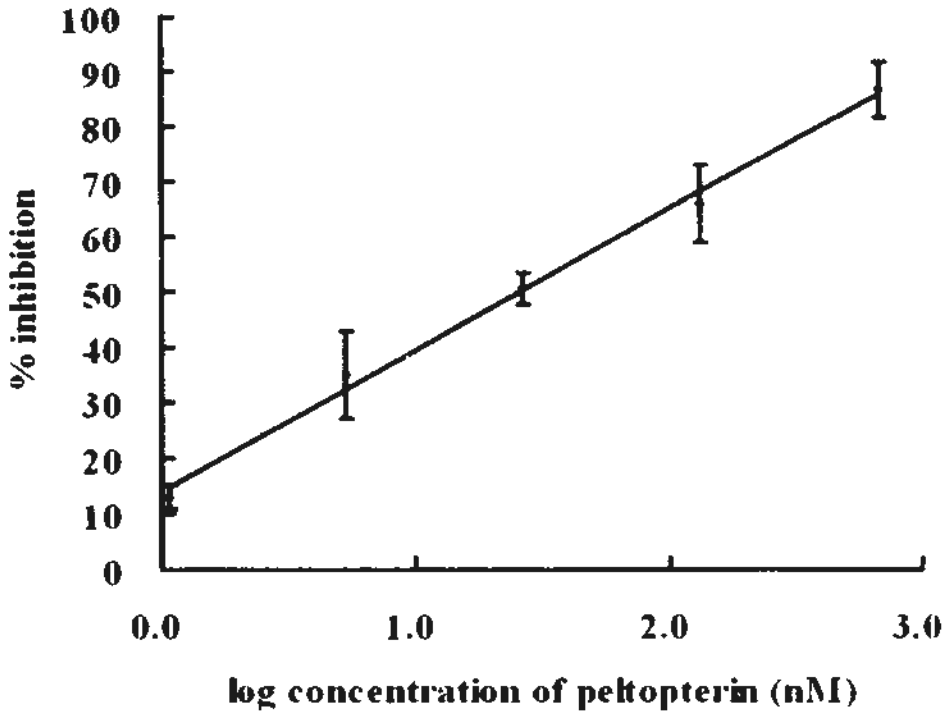


Figure 6.9. Inhibition of HIV-1 reverse transcriptase by peltopterin.

The dose-dependent inhibitory effect of peltopterin on HIV-1 reverse transcriptase was indicated as percent inhibition as compared to a control without the protein. Values are expressed as the mean ± SD (N=3).

Table 6.1. Protein yields (from 180 g fresh *Peltophorum pterocarpum* seeds) and antifungal activity at different stages of purification of peltopterin.

Column	Chromatographic fraction with antifungal activity	Yield (mg)	IC_{50}
-	Crude Extract	1000	-
Q-Sepharose	Q3	220	-
DEAE-cellulose	D2	80	-
Superdex 75	S1	15	0.65 μM

IC_{50} refers to antifungal activity toward *Rhizoctonia solani*.

Table 6.2. N-terminal amino acid sequence of peltopterin.

	Residue no.	Sequence	% Homology	Total no. of residues in protein
Peltopterin	1	FEFKEATIDGIQNF	-	~540
Solanum demissum Amidase	25	FSFKETTIDDIHKA	53	477
Talaromyces stipitatus Amidase	63	FKLEEATIDEIQAE	53	589

6.4. Discussion

Peltopterin manifests an N-terminal sequence that closely resembles those of amidases. It also possesses amidase activity. In general, amide-converting enzymes usually work in a neutral or slightly alkaline pH and within a narrow operating temperature range. The optimum pH and temperature of amidase activity of peltopterin is similar with those of *Geobacillus pallidus* amidase (Makhongela et al., 2007).

Most amidases are polymers. They can be dimeric (human serum N-acetylmuramyl-L-alanine amidase) (De Pauw et al., 1995), tetrameric (*Helicobacter pylori* amidase) (Skouloubris et al., 2001), hexameric (*Geobacillus pallidus* amidase) (Makhongela et al., 2007), or octameric (*Rhodococcus sp.* amides) (Nawaz et al., 1994). There are two cases that monomeric amidases. The molecular mass of monomeric peltopterin (60 kDa) is similar with monomeric *Klebsiella pneumoniae* NCTR 1 amidase (62 kDa) (Nawaz et al., 1996), while monomeric *Ochrobactrum anthropi* SV3 amidase (Komeda and Asano 2000) is much smaller (40 kDa).

Amidases can break down carboxylic acid amides to free carboxylic acids and ammonia. They usually do not have substrate specificity. Soybean amidase hydrolyzes glycopeptides (Kimura and Ohno 1998). *Geobacillus pallidus* amidase

breaks down flouroacetamide, acetamide, and acrylamide (Makhongela et al., 2007). *Brevundimonas diminuta* amidase removes amide group from amino acids (Komeda et al., 2006). *Rhodococcus sp.* amidase hydrolyzes acrylamide, acetamide, butyramide, propionamide, and isobutyramide (Nawaz et al., 1994). Peltopterin can release ammonia from iodoacetamide, urea and acrylamide in the present study. It is worth noting that peltopterin has the properties of urease, which has not been reported in other amidases.

Peltopterin, with antifungal activity, was isolated with a simple chromatographic protocol that entailed only two ion exchange chromatography steps and one gel filtration step. Previous reports show that sometimes an additional affinity chromatography step on Affi-gel blue gel was required for the purification of other antifungal proteins (Wang et al., 2005). The chromatographic behavior of peltopterin on the anion exchangers Q-Sepharose and DEAE cellulose is unique since previously reported antifungal proteins are unadsorbed on anion exchangers (Wang and Ng, 2007) whereas peltopterin is adsorbed. The molecular mass of peltopterin is at the upper end of the molecular mass range exhibited by antifungal proteins. Defensins (Leung et al., 2008) and lipid transfer proteins (Lin et al., 2007) have molecular mass of only a few kilodaltons, while dimeric ribonucleases (Lam and Ng, 2001b) and hemagglutinins (Leung et al., 2008) display a molecular mass of about 60 kDa. It

deserves mention that peltopterin is monomeric and yet has a molecular mass of 60 kDa. The majority of antifungal proteins such as thaumatin-like proteins (Chua et al., 2007), and chitinases (Shenoy et al., 2006), have a molecular mass of approximately 30 kDa. Like defensins, which are pH-stable and thermostable (Leung et al., 2008), the antifungal activity of peltopterin can withstand changes in pH and temperature. However, defensins are peptides whereas peltopterin is a protein. Many antifungal proteins are heat-labile (Wang and Ng, 2002b).

Soybean urease inhibits the growth of the fungi *Colletotrichum musae* and *Curvularia lunata* by interfering with the osmotic balance (Becker-Ritt et al., 2007). Cotton ureases displays antifungal effects against *C. musae*, *C. lunata*, and *Penicillium herguei* by causing plasmolysis and cell wall injuries (Menegassi et al., 2008). These ureases have not been tested for amidase activity using the other substrates employed in the present study. The N-terminal sequences of these ureases are also different from that of peltopterin.

To date, none of the amidases has been reported to have antifungal activity. Peltopterin manifests a potent antifungal action on *R. solani*, though there is no activity against four other fungal species tested. This finding is reminiscent of the observations that the shallot antifungal protein ascalin (Wang and Ng, 2002) and the antifungal lectin from *Capparis edilus* (Lam et al., 2009) exert an antifungal action

on only one out of the several fungi examined. Nevertheless, the antifungal potency of peltopterin is high with an IC_{50} below 1 μM. Its HIV-1 reverse transcriptase inhibitory activity is below 30 nM. The mechanism of its inhibitory action against the retroviral enzyme may be due to protein-protein interaction (Böttcher and Grosse, 1997).

In order to understand the mechanism of antifungal action of peltopterin, Congo red, a dye that strong binds to β-glucans, was used. β-glucan is the major component of chitin in fungal cell wall. The accumulation of chitin is observed when the growing hyphae are inhibited and so a strong Congo red signal is detected. For active growing of hyphae, the level of chitin is low and so the Congo red signal is low. In the Congo red staining assay, the tips of *R. solani* hyphae exposed to peltopterin stained intensely with the dye showing deposition of chitin and consequent arrest in hyphal growth. A similar observation showing that the hyphal tips of rice blast fungus *Magnaporthe grisea* are stained by Congo red after treatment with *Aspergillus giganteus* antifungal protein (Moreno et al., 2006).

The amidase activity of peltopterin plummets to 10 % of the original value after incubation at 100 °C for 15 minutes, while its antifungal activity remains virtually unaltered under the same conditions. The results indicate that the domains responsible for amidase and antifungal activities are different.

Peltopterin exhibits a distinctive amidase-like N-terminal sequence and amidase activity which has not previously been reported for antifungal proteins. All in all, peltopterin is outstanding in several ways, including its amidase-like N-terminal sequence, high molecular mass, pronounced thermostability and pH stability, specificity of antifungal activity, highly potent antifungal, and HIV-1 reverse transcriptase inhibitory activities. The fact that peltopterin demonstrates antifungal, and anti-HIV reverse transcriptase is in line with its role as a defense protein. Ribosome-inactivating proteins, a class of pathogenesis related proteins which may have antifungal activity, have similar biological activities, including HIV-1 reverse transcriptase inhibitory, antifungal, and antiproliferative activities (Lam and Ng, 2001a). It has been shown that the different RIPs are the consequence of different mechanisms and that some of the activities may be selectively affected without changing the other activities (Lee-Huang et al., 1994). Likewise, in the present study, the amidase and antifungal activities of peltopterin are differentially affected by changes in the ambient pH and temperature, indicating that regulation of these activities by different domains.

The biochemical components in seeds are mainly carbohydrates, oil, proteins and small molecules. They are all important during germination. The proteins may serve as storage proteins for germination. They may also serve as defense proteins against the pathogens, like bacteria, fungi and insects. Five defense proteins were purified in this project and they are *Capparis spinosa* lectin, *Hibiscus mutabilis* lectin *Phaseolus vulgaris* cv French bean 35 hemagglutinin, *Passiflora edulis* antifungal protein (passiflin), *Peltophorum pterocarpum* antifungal protein (peltopterin), their biological activities were also characterized.

7.1. Chromatographic Behavior of Purified Proteins

Different chromatographic methods were used to purify defense proteins, including anion exchange chromatography (Q-Sepharose, DEAE-cellulose and CIM-QA), cation exchange chromatography (SP-Sepharose), hydrophobic chromatography (phenyl-Sepharose), affinity chromatography (Blue-Sepharose), and gel filtration (Superdex 75 and Superdex 200) (Table 7.1). There is no specific protocol for purification of defense proteins. Amongst all of them, gel filtration was used frequently. It was used in the last step to remove small molecules. Anion exchange chromatography was also commonly employed. It is interesting that the purification of *H. mutabilis* lectin depended mainly on gel filtration (due to unable to desorb or unable to absorb on a large number of chromatographic medium), which was different from other lectins that isolated with Blue-Sepharose and anion exchangers.

The yields of purified proteins were generally less than 10 mg per 100 g seed. The protein yield was relatively low because the sources of plants were healthy so as the expressions of defense proteins were low (van Loon et al., 2006). The unexceptional high yield of lectin from French bean 35 (1100 mg/100g seed) is probably due to its role as a storage protein.

Table 7.1. Summary of the chromatographic behaviors of proteins purified in this study.

Protein	1st chromato-graphic medium	2nd chromato-graphic medium	3rd chromato-graphic medium	4th chromato-graphic medium	Yield (mg/100g seeds)
C. spinosa lectin	DEAE-cellulose (adsorbed)	SP-Sepharose (adsorbed)	CIM-QA (adsorbed)	Superdex 75	3.4
H. mutabilis lectin	SP-Sepharose (unadsorbed)	Superdex 75	Superdex 200	-	4.0
French bean hemagglutinin	Affi-Gel Blue gel (adsorbed)	Q-Sepharose (adsorbed)	Superdex 75	-	1100
Passiflin	Q-Sepharose (adsorbed)	Phenyl-Sepharose (adsorbed)	DEAE-cellulose (adsorbed)	Superdex 75	5.0
Peltopterin	Q-Sepharose (adsorbed)	DEAE-cellulose (adsorbed)	Superdex 75	-	8.3

7.2. Physiochemical Properties of Purified Proteins

The physiochemical properties of purified proteins are summarized in Table 7.2. The molecular mass of both *C. spinosa* lectin and French bean 35 hemagglutinin is 62 kDa. Both of them are dimeric, which is similar with most lectins/hemagglutinins. *H. mutabilis* lectin is hexameric with a molecular mass of 150 kDa. The N-terminal amino acid sequence of French bean 35 hemagglutinin is similar with other *P. vulgaris* lectins/hemagglutinins, while those of *C. spinosa* lectin and *H. mutabilis* lectin are different from other known lectins.

The sugar specificities of lectins purified in this study are very different. *C. spinosa* lectin is a superlectin because it can be inhibited by 6 sugars, including raffinose, α-lactose, rhamnose, D(+)galactose, L(+)arabinose and D(+)glucosamine. *H. mutabilis* can be inhibited by galactonic acid, which is the first galactonic acid-binding lectin while there is no sugar specificity found in French bean hemagglutinin.

Two high-molecular-mass antifungal proteins, passiflin and peltopterin, were purified from *P. edulis* (67 kDa) and *P. pterocarpum* (60 kDa). Unlike most antifungal proteins, passiflin is dimeric. Passiflin possesses a β-lactoglobulin-like N-terminal sequence, while peltopterin shows an amidase-like N-terminal sequence. No proteins with such N-terminal sequences display antifungal activity.

The thermostability of lectins and hemagglutinin isolated in this study is low, up to 50 °C, which is probably due to their dimeric nature. The pH stability of both *H. mutabilis* lectin and French bean 35 hemagglutinin is low, unlike *C. spinosa* lectin, which is stable from pH 1 – 12. The thermostability and pH stability of passiflin were not determined due to the low yield, while peltopterin is the most robust, with full activity in the termperature rang of 0 °C – 100 °C and the pH range of 0 – 14.

Table 7.2. Summary of the physiochemical properties of proteins purified in this study.

Protein (origin)	Molecular mass (kDa)	Number of subunits	Thermostability (°C)	pH stability
Lectin (*Capparis spinosa*)	62	2	0 – 40	1 – 12
Lectin (*Hibiscus mutabilis*)	150	6	0 – 50	4 – 7
Hemagglutinin (*Phaseolus vulgaris* cv French bean 35)	62	2	0 – 50	6 - 8
Antifungal protein (*Passiflora edulis*)	67	2	Not determined	Not determined
Antifungal protein (*Peltophorum pterocarpum*)	60	1	0 – 100	0 – 14

7.3. Biological activities of purified proteins

The biological properties of purified proteins are summarized in Table 7.3. Besides hemagglutinating activity, the purified lectins and hemagglutinin showed different degree of antiproliferative activity. *C. spinosa* lectin showed potent inhibitory effect on both HepG2 and MCF-7 cells, while *H. mutabilis* lectin exerted a weak effect on both cells. French bean hemagglutinin displayed specific antiproliferative activity against MCF-7 cells and it was confirmed that apoptosis is the mechanism involved. All of them possessed the inhibitory potent effect on HIV-1 reverse transcriptase. And *C. spinosa* lectin and *H. mutabilis* lectin had specific antifungal activity against *V. mali*.

Both passiflin and peltopterin showed particular antifungal activity toward *R. solani*. Passiflin exhibited antiproliferative effect toward HepG2 cells, but not toward MCF-7 cells. Peltopterin is totally devoid of such activity. On the other hand, peltopterin demonstrated a potent inhibitory effect on HIV-1 reverse transcriptase activity while passiflin lacked such effect. Peltopterin is an amidase, which can break down ioacetamide, urea and acrylamide to free carboxylic acids and ammonia.

Table 7.3. Summary of the biological properties of proteins purified in this study.

Protein (origin)	Antiproliferative activity toward cancer cells (IC_{50})	Antifungal activity (IC_{50})	Anti-HIV-1 reverse transcriptase activity (IC_{50})
Lectin (*Capparis spinosa*)	HepG2: 2μM MCF-7: 2μM	*V. mali*: 18 μM	0.28 μM
Lectin (*Hibiscus mutabilis*)	HepG2: 100μM MCF-7: (IC_{40}) 100μM	Inactive	0.2 μM
Hemagglutinin (*Phaseolus vulgaris* cv French bean 35)	HepG2: 100μM MCF-7: 2μM WRL68: Inactive	*V. mali*: 10 μM	2 μM
Antifungal protein (*Passiflora edulis*)	HepG2: 15 μM MCF-7: Inactive	*R. solani*: 16 μM	Inactive
Antifungal protein (*Peltophorum pterocarpum*)	HepG2: Inactive MCF-7: Inactive	*R. solani*: 0.65 μM	27 nM

7.4. Future Perspectives and Conclusion

In this investigation, two lectins, one hemagglutinin and two antifungal proteins were purified from five different species, *C. spinosa, H. mutabilis, P. vulgaris* cv French bean 35, *P. edulis* and *P. pterocarpum*. Different classes of plant defense proteins may be synthesized by different organisms. Lectins or hemagglutinins are abundant proteins in grain legumes, which is the same in French bean 35. On the other hand, lectins are present at lower levels (*C. spinosa* and *H. mutabilis*) or absent (*P. edulis* and *P. pterocarpum*) in other species. There is an overlap in the spectra of biological activities of these plant defense proteins, although the potency of each type of biological activity may differ from one protein to another. The ability of each of the aforementioned plant defense proteins to exert a protective action against pathogen may be reflected in their antifungal, HIV-1 reverse transcriptase-inhibiting and antiproliferative activities.

The application of genetic modification to produce recombinant proteins of high nutritional or therapeutic value in the legumes has been increasing because of a better understanding about the role and mechanisms of action of proteins/peptides. One example is the genetic modification with up to four ovokinin peptides introduced into the soybean protein which resulted in a 200-fold more potent anti-hypertensive effect than ovalbumin (Onishi et al., 2004). This technique can introduce proteins or

peptides into traditional plant foods. The genetically modified foods are expected to have higher acceptance by consumers, due to the beneficial effects on human health. The transgenic plant with antifungal proteins can increase the resistance of plants to infections of pathogens and increase the crop yield.

Pharmaceutical application of purified proteins is feasible. Lectins and hemagglutinins have been investigated as antiviral (Balzarini et al., 1992) and antitumor reagents (Yang et al., 2008). The discovery of the apoptotic mechanism of French bean 35 hemagglutinin has made it a potential antitumor drug. The purified proteins with an inhibitory effect on HIV-1 reverse transcriptase, which is one of the enzymes crucial in retrovirus replication (De Clercq, 2004), may find application for treatment of AIDS patients.

The discovery of more lectins, hemagglutinin and antifungal proteins of therapeutic potential in the future and elucidation of their mechanism of action may be beneficial to human health.

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