LECTINS FROM TROPICAL MUSHROOMS

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จากการเก็บรวบรวมตัวอย่างดอกเห็ดทั้งสิ้น 218 ตัวอย่าง ทั้งที่เจริญในธรรมชาติและที่จำหน่ายในท้องตลาดโดยเฉพาะอย่างยิ่งในภาคตะวันออกเฉียงเหนือของประเทศไทย เมื่อเตรียมสารสกัดหยาบจากดอกเห็ดเหล่านั้นและตรวจหาสารเล็กตินที่สะสมในดอกเห็ดด้วยปฏิกิริยาการจับกลุ่มของเซลล์เม็ดเลือดแดงของคน (เลือดหมู่ เอ บี และโอ) และสัตว์ (ห่าน กระต่าย หนู และแกะ) พบว่ามากกว่า 88 เปอร์เซ็นต์ของสารสกัดเล็กตินเกิดปฏิกิริยาการจับกลุ่มกับเซลล์เม็ดเลือดแดงของหนู เห็ดที่มีเล็กตินสูงอยู่ในวงศ์ Agaricaceae, Cantharellaceae, Pleurotaceae และ Tricholomataceae และเมื่อคัดเลือกสารสกัดหยาบจากเห็ด 10 ตัวอย่าง ไปตรวจสอบฤทธิ์ในการยับยั้งการเจริญของจุลินทรีย์ พบว่ามีสารสกัดจาก *Stereum* sp. ML005, *Microporus* sp. ML006 และ *Schizophyllum commune* ML078 มีฤทธิ์สูงในการยับยั้งการเจริญของ *Staphylococcus aureus*, *Aspergillus niger* และ *Bacillus cereus*, และ *Aspergillus niger* ตามลำดับ และเมื่อนำสารสกัดเล็กตินจากเห็ดที่คัดเลือกไปทดสอบความเป็นพิษกับเซลล์มะเร็งของคนพบว่าสารสกัดจาก *Amanita* sect. *Vaginatae* ML020, *Lentinus* sp. ML055, *S. commune* ML078 และ *Strobilomyces mollis* ML034 มีผลต้านทานเซลล์มะเร็งเยื่อบุช่องปากได้ดีและสารสกัดจาก ML055, ML020, *Macrolepiota* sp. ML106 และ *Marasmius* sp. ML071 มีผลต้านทานเซลล์มะเร็งปากมดลูกได้ดี จึงเลือกเห็ด ML078 มาสกัดและศึกษาสารเล็กติน ซึ่งพบว่าสารเล็กตินบริสุทธิ์จากเห็ดชนิดนี้เป็นสารไกลโคโปรตีน มีน้ำหนักโมเลกุล 31 กิโลดาลตัน มีความจำเพาะต่อน้ำตาลกาแล็กโทส มีความเสถียรต่อความร้อนที่อุณหภูมิ 55 องศาเซลเซียส นาน 30 นาที และเสถียรต่อค่าความเป็นกรดด่างในช่วงพีเอช 3 ถึง 10 ตลอดระยะเวลา 18 ชั่วโมงที่ทดสอบ เมื่อวิเคราะห์หาลำดับของกรดอะมิโนและเปรียบเทียบกับเล็กตินชนิดอื่นที่มีรายงานแล้ว พบว่าลำดับกรดอะมิโนของเล็กตินนี้มีเปอร์เซ็นต์ความเหมือน 66 และ 53 เปอร์เซ็นต์กับเล็กตินของเห็ดสกุล *Marasmius* และ *Polyporus* ตามลำดับ และสามารถปลูกผลึกสารเล็กตินจากเห็ด ML078 ได้สำเร็จ โดยผลึกเล็กตินที่ได้ให้แบบแผนการกระจายรังสีเอกซ์ที่มีการแจกแจงรายละเอียด 3.8 อังสตรอม ซึ่งเป็นประโยชน์ต่อการศึกษาสารเล็กตินและโครงสร้างของสารในเชิงลึกต่อไป

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ลายมือชื่อนักศึกษา พจนา ชุ่มขุนทด
ลายมือชื่ออาจารย์ที่ปรึกษา
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LECTIN/TROPICAL MUSHROOM/CRYSTALLIZATION/
X-RAY CRYSTALLOGRAPHY/CYTOTOXICITY

A total of 218 mushroom specimens were collected from natural habitats and local markets, particularly in the Northeastern Thailand. Accumulations of lectins in crude extracts of these mushroom specimens were detected by hemagglutination assay using human (A, B, and O blood groups) and animal (goose, rabbit, rat, and sheep) red blood cells. It was found that more than 88% of mushroom extracts predominantly performed hemagglutinating for rat red blood cells. The high incidence of lectin accumulations was observed in mushroom specimens belonging to families; Agaricaceae, Cantharellaceae, Pleurotaceae, and Tricholomataceae. Then crude extracts of ten selected mushrooms were submitted to the antimicrobial activity assay. It revealed that mushroom extracts from *Stereum* sp. ML005, *Microporus* sp. ML006, and *Schizophyllum commune* ML078 had high activity against *Staphylococcus aureus*, *Aspergillus niger* and *Bacillus cereus*, and *Aspergillus niger* respectively. Some selected extracts were also submitted to cytotoxicity test against cancer cells. The most powerful extracts with anti-human epidermoid carcinoma cells were from *Amanita* sect. *Vaginatae* ML020, *Lentinus* sp. ML055, *S. commune* ML078, and *Strobilomyces mollis* ML034 respectively. The high activity against human cervical carcinoma cells was with extracts from ML055, ML020, *Macrolepiota* sp. ML106,

and *Maramius* sp. ML071. The mushroom ML078 was finally selected for a detailed study of its lectin. The purified lectin, which is a glycoprotein, had molecular weight of 31 kDa. It showed specificity towards galactose. The lectin was stable at 55°C for 30 min and also with pH 3-10 for 18-h test. For amino sequence analysis, amino acid sequence of the lectin was compared for their homology with other lectins that have been reported. The lectin showed percent similarity with lectins from mushroom genera *Maramius* and *Polyporus* at 66% and 53% respectively. Small crystals of the *Schizophyllum commune* lectin were successfully grown, which gave an X-ray diffraction pattern, which diffracted to approximately 3.8 Å resolution. This information is very useful for further structural study of the lectin.

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Podjana Chumkhunthod

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LIST OF ABBREVIATIONS

Å	Angstroms
cm	Centimeter
ºC	degree Celsius
CRD	Carbohydrate recognition domain
ConA	Concanavalin A
DAP	Diaminopropane
DNA	Deoxyrobonucleic acid
D.W.	Distilled water
et al.	et alia (and others)
e.g.	for example
etc.	et cetera, and others
g	Gram
h	Hour
HA	Influenza virus hemagglutination
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
kDa	KiloDaltons
min	Minute
mg	Milligram
ml	Milliliter
mM	Millimolar

LIST OF ABBREVIATIONS (Continued)

Mw	Molecular weight
%	Percentage
pp.	Page
PBS	Phosphate buffer saline
PFG	Polyethylene glycol
l	Liter
RIPs	Ribosome inactivating proteins
SDS-PAGE	Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SRS	Synchrotron radiation source
µg	Microgram
µl	Microliter
U.K.	United Kingdom

CHAPTER I

INTRODUCTION

1.1 Overview of mushroom lectins and their significance

Lectins are proteins or glycoproteins of non-immune origin that specifically and reversibly bind to carbohydrates of glycoconjugates (Rini, 1995; Lindhorst, 2000). These proteins are ubiquitous in nature, and occur in animals, bacteria, fungi, plants and viruses (Lis and Sharon, 1998; Mo *et al.*, 2000). Most lectins play a crucial role in diverse biological processes, particularly in host defense mechanisms, inflammation, and metastasis (Imberty *et al.*, 2000). Owing to their binding specificities, lectins are employed in a number of biochemical and clinical research areas (Gilboa-Garber *et al.*, 1997). Many of the animal, plant, and microorganism lectins with known carbohydrate specificities have been engaged in commercialization (Pemberton, 1994). *Agaricus bisporus* lectin is the most well characterized lectin of fungal origin (Wang *et al.*, 1998b).

In recent years, mushroom lectins have become of more interest, mainly due to the discovery of some of these lectins exhibiting potent biological activities (Mo *et al.*, 2000), for example, *Agaricus bisporus* lectin shows its antiproliferation activity against human colon cancer cell lines HT29 and breast cancer cell lines (MCF-7) (Yu *et al.*, 1999). *Volvariella volvacea* lectin possesses antitumor activity to sarcoma S-180 cells (Wang, 1998b). *Tricholoma mongolicum* lectin inhibits mouse mastocytoma P815 cells *in vitro* and sarcoma S-180 cells *in vivo* (Wang *et al.*, 1995)

Grifola frondasa lectin is also cytotoxic to HeLa cells (Guillot and Konska, 1997). The lectins from *Volvariella volvacea, Boletus satanas* Lenz, *Flammulina velutipes*, *Ganderma lucidum*, *Lentinus edodes*, and *Agrocybe cylindracea* exhibit potent mitogenic activities (Ho *et al.*, 2004). In addition, some lectins including mushroom lectins express other potential activities such as immunoenhancing, vasorelaxing, hypotensive, and antimicrobial activities (Gozia *et al.*, 1993; Verheyden *et al.*, 1995; Wang *et al.*, 1998b). These clearly indicate that mushroom lectins might be employed as drugs or therapeutic reagents for pharmaceutics; mushrooms have now become a valuable source of lectins for drug discovery.

In view of the great diversity of mushrooms, which provides a high probability for lectin exploitation (Doyle and Slifkin, 1994), Thailand, a tropical country, is considered to be one of the most potential countries for mushroom lectin investigation because of the extremely high varieties of macrofungi. Thailand might show the highest diversities in Asia due to the natural geography of the country (Thaithatgoon, 1998; Walting, 1998). At least 90 genera of mushrooms have been reported to be found in National Forests of Thailand including Doi Suthep National Forest, Doi Inthanon National Forest, Khao Yai National Park (Walting, 1998), Tup Lan National Park (Rodtong and Teaumroong, 2000), Nong-rawieng Plant Genetics Forest (Rodtong *et al.*, 1998), Khao Kicha Koot National Park (Klingesorn *et al.*, 1998a), and Khao Soi Dao Wildlife Sanctuary (Klingesorn *et al.*, 1998b). The common families found belong to *Agaricaceae, Coriolaceae, Ganodermataceae, Geastraceae, Hygrophoraceae, Hymenochaetaceae, Lycoperdaceae, Polyporaceae, Russulaceae, Tricholomataceae.*

In this study, lectins from tropical mushrooms which were collected from forests

in Thailand, particularly in nearby Nakhon Ratchasima Province were intensively determined and screened for their biological activities. The mushroom lectin with potent antimicrobial activities was purified and characterized. The information on the tropical mushroom lectin will be useful for future research work on the rational structural based-drug design.

1.2 Research objectives

1) To determine the variety and quantity of lectins accumulated in the tropical mushroom species from the Northeastern, Central, and Western Thailand using hemagglutination assay.

2) To isolate, purify, and characterize the selected mushroom lectin with novel properties.

3) To try to crystallize the selected mushroom lectins for structural study.

1.3 Expected results

1) The information of lectins distribution in the tropical mushroom regarding to blood group specificity in the Northeastern, Central, and Western Thailand that will be useful for a long-term research study.

2) The appropriated isolation and purification techniques used for the tropical mushroom investigation and biochemical characteristic data of the lectin that lead to the future novel biochemical and medical applications or even the evolutionary study of the lectin family.

3).The data obtained from the crystallization of the tropical mushroom lectin and preliminary structural study that will be beneficial for further three dimensional

structure studies and also specific recognition mechanism study of the mushroom lectins.

CHAPTER II

LITERATURE REVIEW

2.1 Lectins

Lectins are multivalent proteins or glycoproteins of non-immune origin that bind specifically and reversibly to carbohydrates, resulting in agglutination of cells or precipitation of glycoconjugates (Rini, 1995; Mo *et al.*, 2000). The first lectin discovered was ricin, a very toxic protein from castor bean (*Ricinus communis*), was extracted by Stillmark in 1888. Apparently, the best known lectin is concanavalin A, a protein of the jack bean that was originally crystallized by Sumner in 1919. It is a typical of the family of legume lectins. Generally, lectins contain one or more sites specific to carbohydrate binding called carbohydrate recognition domains (CRD) (Rini, 1995; Lindhorst, 2000).

Lectins may interact with carbohydrates through hydrogen bonds, metal coordination, Van der Waals, and hydrophobic interactions (Weis and Drickamer, 1996; Elgavish and Shaanan, 1997). Basically, hydroxyl groups on sugar molecules can serve as both a donor and an acceptor to cooperate in hydrogen bonds (Elgavish and Shaanan, 1997). Hemagglutination activity is a major property of lectins and has been widely used for their detection and characterization (Lis and Sharon, 1998; Pusztai, 1991). However, several lectins possess only one binding site for specific sugars, therefore they cannot agglutinate red blood cells (Peumans, 1995a; 1995b; Sharon and Lis, 1989).

Based upon the overall structures of plant lectins, three major types of lectins are classified namely “merolectins,” “hololectins,” and “chimerolectins” (Peumans and Van Damme, 1995). Merolectins are small, single polypeptide proteins, which have a single

carbohydrate-binding domain. They are monovalent and incapable of precipitating glycoconjugates or agglutinating cells. Examples of this group are hevein (Van Parijs *et al.*, 1991) and the monomeric Man-binding proteins from orchids (Pusztai, 1991). Hololectins also have similar structural pattern as merolectin, but contain two or more identical carbohydrate-binding domains. Obviously, the majority of all known lectins belong to hololectins (Peumans and Van Damme, 1995), which have multiple binding sites, and are capable of agglutinating cells or precipitating glycoconjugates, and be defined as haemagglutinins (Sharon and Lis, 1989; Pusztai, 1991). These plant lectin structures are shown in Figure 1 below.

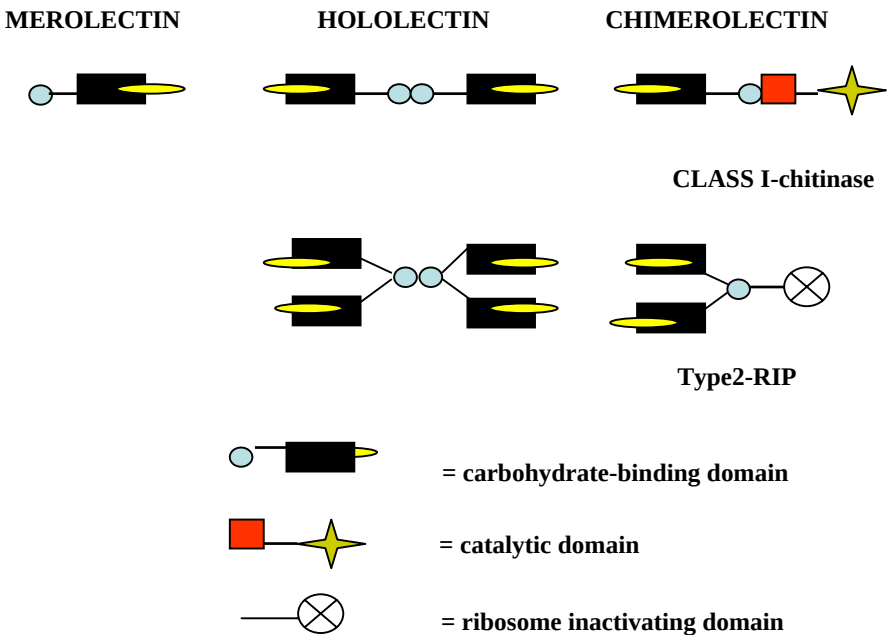

Figure 1. Schematic representation of the three types of plant lectins: merolectins, hololectins, and chimerolectins.

Modified from: Peumans and Van Damme (1995a).

Chimerolectins are a group of fusion proteins possessing a carbohydrate-binding domain, which have an additional activity that acts independently of carbohydrate-binding domain (Peumans and Van Damme, 1995). Generally, chimerolectins behave in the same manner as merolectins or hololectins in respect to the number of sugar-binding sites. For example, type 2 RIPs with two carbohydrate-binding sites on their B chain (e.g. ricin) agglutinate cells (Barbieri *et al.*, 1993), whereas class I plant chitinases with a single chitin-binding domain do not (Collinge *et al.*, 1993).

Lectins which are glycoproteins are synthesized at the endoplasmic recticulum (ER) of eukaryotic cells and consequently modified at the Golgi complex apparatus by the glycosylational process (Fukuda and Hindsgaul, 1994; Hauri *et al.*, 2000). According to chemical linkages on molecules, two major structures of glycoproteins, N-glycoproteins and O-glycoproteins, are found (Comer and Hart, 2000; Spiro, 2000). In contrast to the biosynthesis of O-glycoproteins, in which their carbohydrate, moieties are built-up stepwise directly on the proteins at serine and threonine residues. The biosynthesis of N-glycoproteins needs carbohydrate precursors for assembling carbohydrate on a lipid carrier before being transferred to the nascent polypeptide at asparagine residues (Lindhorst, 2000; Spiro, 2000).

2.2 Lectin sources

Lectins are widely distributed in nature, and occur in diverse organisms ranging from plants, animals, fungi, bacteria, and viruses (Mo *et al.*, 2000). A brief overview is given below.

2.2.1 Plants

Legumes and monocots are major sources of plant lectins that have been widely studied (Wood *et al.*, 1999). Plant lectins can be classified into four major families of

structurally and evolutionary related proteins: legume lectins, type 2 ribosome-inactivating proteins, chitin-binding lectins, and monocot mannose-binding lectins (Van Damme *et al.*, 1999; Wright *et al.*, 1999a, 1999b). Three other small lectin families (Cucurbitaceae phloem lectins, amaranthins, and jacalin-related lectins) have also been characterized (Van Damme *et al.*, 1999).

Legume lectins represent the largest and most thoroughly studied family of plant lectins. They have been isolated from seeds, stem, and bark of legumes (Imberty *et al.*, 2000). The best known legume lectins are phytohemagglutinin (PHA) from red kidney bean, soybean (SBA), jackbean (concanavalin A), peanut lectin (PNA), and pea (PSL) (Lis and Sharon, 1998).

Type 2 ribosome-inactivating proteins consist of the toxic A subunit and Gal/GalNAc binding subunit of B chain. Whereas the A chain has RNA glycosidase activity, the B chain is responsible for binding to the target cell surface and helping in the internalization of the whole protein into cell membrane (Kaku *et al.*, 1996, Wood *et al.*, 1999). Ricin from seeds of *Ricinus communis*, the first plant lectin, is an example (Sphyris *et al.*, 1995; Lisgarten *et al.*, 1999).

Chitin-binding lectins containing hevein domains have been prevalently found in cereal. Examples are wheat germ agglutinin, pokeweed mitogen, rice, rye, and barley lectins (Lis and Sharon, 1998; Wright *et al.*, 1999b; Wood *et al.*, 1999).

Monocot mannose-binding lectins were first reported from the snowdrop (*Galanthus nivalis*) (Van Damme *et al.*, 1997b). Later several lectins have been extracted and intensively characterized from several monocot families: Alliaceae, Amaryllidaceae, Araceae, Bromeliaceae, Iridaceae, Liliaceae, and Orchidaceae (Wright *et al.*, 1999b, Wood *et al.*, 1999). For example, *Narcissus pseudonarcissus* (daffodil) and *Scilla campanulata* (bluebell) lectins have been recently reported (Sauerborn *et al.*, 1999; Wood

et al., 1999).

2.2.2 Animals

Animal lectins are found in diverse species of vertebrates including mammals as well as invertebrates, such as snails, crabs, and molluscs (Miarons and Fresno, 2000; Dodd and Drickamer, 2001). They are classified into two major groups: C-type lectins and S-type lectins (Frits and Gary, 2000). C-type lectins are calcium-dependent lectins, which are the most diverse family of animal lectins. The carbohydrate-binding activity of the lectins depends not only on the presence of Ca^{2+} but also on the function of carbohydrate recognition domains (CRD), and their structures are highly conserved among the family (Dodd and Drickamer, 2001). Moreover, C-type lectins are composed of two major classes of selectins and collectins. Selectins are transmembrane C-type lectins known as a family of cell adhesion molecules. Collectins are a group of oligomeric C-type animal lectins, which consist of three main types of serum lectins (mannose-binding protein (MBP), conglutinin, and collectin-43 (CL-43)) and two pulmonary surfactant proteins (SP-A and SP-D) (Matsushita *et al.*, 1996).

S-type lectins are soluble and β-galactose specific binding proteins normally known as galectins (Frits and Gary, 2000; Gitt *et al.*, 1995). Galectins are found in both vertebrates and invertebrates including nematodes, insects, sponges, and mushrooms (Hirabayashi, www, 1997b). Annexins, another lectin that comprise a family of calcium- and phospholipid-binding proteins have also been reported in plants and animals (Kojima *et al.*, 1996). Annexins bind to phosphatidylinositol, which are rich in the inner leaflet of plasma membrane and hardly appear on cell surface. Additionally, some animal lectins known as calnexin and calreticulin are localized in the endoplasmic reticulum (ER). They

operate as chaperones in the quality control of newly synthesized glycoproteins along the secretary pathway (Hauri *et al.*, 2000; Dodd and Drickamer, 2001).

2.2.3 Microorganisms

a) Viruses

Hughes *et al.* (2001) reported that influenza A viruses possess two surface proteins, hemagglutinin (HA) and neuraminidase (NA). The HA protein, a trimeric type I membrane protein, is a sialyloligosaccharide binding protein. Lis and Sharon (1997) demonstrated that murine polyoma virus, a nonenveloped, symmetrical particle, with circular and double-stranded DNA, was another source of viral lectins. The outer shell of the virion contains 360 copies of the viral protein (VP1) arranged in pentamers. Each subunit of the protein has two antiparallel β sheets, which resembles the lectin fold.

b) Bacteria

Intensive studies of lectins in *Eschericia coli, Streptococcus sobrinus*, *S. cricetus,* and *Pseudomonas aeruginosa*, have been documented (Sharon and Lis, 1989). Some streptococci, such as *Streptococcus sobrinus* and *Streptococcus cricetus*, possess glucan-binding lectins that are associated on their cell surfaces as well as secreted out of cells that can be detected in the culture medium (Wu *et al.*, 1995).

c) Fungi

Several fungi can express high levels of saline-soluble and low molecular weight lectins. The parasitic fungus, *Arthrobotrys oligospora*, contains a multispecific lectin that can bind to fetuin and mucins (Rosen *et al.*, 1996).

Recently, fungal lectins especially from either mushrooms or filamentous fungi have been the focus of research. The high content of lectins in mushrooms has been detected in diverse species of genera *Lactarius*, *Russula*, *Boletus*, *Phallus*, and

Hygrophorus (Guillot and Konska, 1997; Wang *et al.*, 1998b). Hemolysins are found in families *Hygrophoraceae* and *Strophariaceae*; in genera *Amanita*, *Mycena*, *Agrocybe, Oudemansiella, Hebeloma,* and *Gymnopilus*; and in many ascomycetes (Guillot and Konska, 1997). Guillot and Konska (1997) summarized that lectins were localized on caps, stipes, and mycelia of mushrooms, and variations in lectin contents occurred upon their carpophore ages and the time and place of harvest. Lectins from *Pleurotus cornucopiae* (Oguri *et al.*, 1996) and *Tricholoma mongolicum* have been reported to be isolated from mycelia (Wang *et al.*, 1998a). Surprisingly, the lectin from *Pleurotus cornucopiae* mycelium could be detected only in dikaryotic, not in monokaryotic mycelium, and it disappeared during the formation of fruit bodies (Oguri *et al.*, 1996).

2.3 Physiological roles of lectins in nature

Lectins are involved in numerous recognition events at the molecular or cellular level such as host defense mechanisms, cell-cell interactions, and protein targeting within cells (Peumans and Van Damme, 1995; Elgavish and Shaanan, 1997).

2.3.1 In plants

Even though plant lectins have been extensively studied, the role of plant lectins is still not well understood. However, it has been proposed that plant lectins act as storage proteins (The storage of nitrogen mainly in germinated seed) and play a crucial role as host defense proteins (Janzens *et al.*, 1976; Peumans and Van Damme, 1995). The toxicity of various plant lectins for animals and their growth inhibitory effect on fungi are the basis assumption that they function in the defense of plants against phytopathogenic fungi and predatory animals (Lis and Sharon, 1998; Gatehouse *et al.*, 1995). Most lectins are stable at wide pH range and are resistant to animal and insect proteases (Peumans and Van Damme, 1995). Since it has been observed that elderberry (*Sambucus nigra*) and

black locust (*Ribinia pseudoacacia*) are never been attacked by any animal, the bark lectins of both plants have been studied and they found that the lectins showed toxicity effects to animal. Additionally, type 2 RIPs exhibit an inhibitory effect against plant viruses (Hartley *et al.*, 1996; Van Damme *et al.*, 1997). Furthermore, plant lectins are also thought to have interactions with soil bacteria for the purpose of symbiosis establishment (Peumans and Van Damme, 1995). Since the legume root lectins have been reported that may be involved in symbiosis between leguminous plants, e.g. pea (*Pisum sativum*), sweet pea (*Lathyrus ochrus*), lentil (*Lens culinaris*), and vetch (*Vicia faba*); and nitrogen-fixing bacteria (mainly *Rhizobium* and *Bradyrhizabium*) (Guillot and Konska, 1997; Frits and Gary, 2000; Ngai and Ng, 2004).

2.3.2 In animals

The animal lectins are believed to function in cell-cell interactions, and endocytosis mechanism processes (Elgavish and Shaanan, 1997; Matsushita *et al.*, 1996). The selectins, which are another family of C-type lectins, mediate the adhesive interaction between leukocytes and endothelial cells of blood vessels during leukocyte extravasations. They are involved in the controlling of leukocyte trafficking at inflammation sites and the migration (homing) of lymphocytes to specific lymphoid organs (Figure 2) (Lis and Sharon, 1998; Imai, www, 1998). Collectins, which have a collagen-like domain and play a potential role in resistance to bacterial and viral infections by acting as neutralizing antibody or through killing mediated by the complement system as well as opsonization (Matsushita *et al.*, 1996; Ohtani *et al.*, 1999). The functions of intracellular annexins include the regulation of phospholipase A2 activity due to the sequestration of substrate phospholipids, and the involvement in vesicular transport and trafficking, endocytosis, and exocytosis (Kojima *et al.*, 1996;

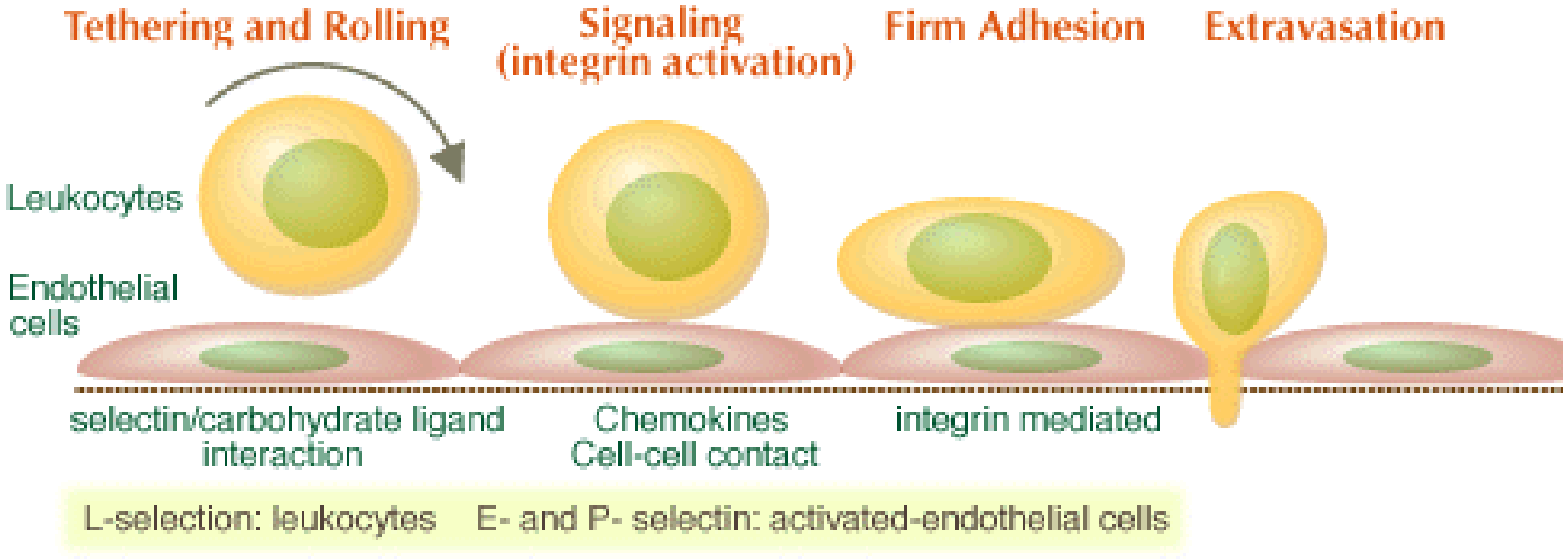

Figure 2. Adhesion and signaling events during leukocyte extravasations: inflammation and lymphocyte homing.

Source: www.glycoforum.gr.jp/science/word/lectin/LEA05E.html

Kojima, www, 1997). Some of animal lectin structures and functions are summarized in Table 1.

Table 1. Types of carbohydrate-recognition domains (CRDs).

Type	Structure	Typical ligand	Example of function
Calnexin	β-sandwich	Glc_1Man_g	Protein sorting in the endoplasmic reticulum
M-type	α-helical barrel	Man_8	Endoplasmic reticulum-associated protein degradation
L-type	β-sandwich	Man_{5-9}	Protein sorting post-in the endoplasmic reticulum
P-type	Unique β-rich structure	Man 6-phosphate	Protein sorting post-Golgi
C-type	Unique mixed α/β structure	Various	Cell adhesion (selectins) Glycoprotein clearance Innate immunity (collectins)
Galectins	β-sandwich	ß-galactosides	Glycan cross-linking in the extracellular matrix
I-type	Immuloglobulin superfamily	Sialic acid	Cell adhesion (Siglecs)
R-type	β-trefoil	Various	Enzyme targeting Glycoprotein hormone turnover

Source: Taylor and Drickamer (2003)

2.3.3 In viruses

The hemagglutinin (HA) protein from influenza viruses is responsible for fusion between viruses and host cell membranes, following virion internalization by endocytosis. The HA protein has also been identified as the first viral lectin with lymphocyte-activating properties (Rott *et al.*, 1996). The neuraminidase (NA) is a sialidase that cleaves terminal sialic acid residues from the glycoconjugates of host cells.

2.3.4 In bacteria

Bacterial lectins play a key role in the initiation of infection by mediating bacterial adherence to epithelial of host cells. The FimH adhesin (a subunit of fimbrial proteins) of *E. coli* has been reported to be responsible for promoting the adherence of the bacteria to human urinary epithelial cells (Mirelman, 1986; Thankavel *et al.*, 1997).

However, the lectins on the cell surface of bacteria may also bind to sugars on phagocytic cells and activate of phagocytosis of bacteria resulting in cell death (Sharon and Lis, 1989). Two lectins (PA-IL and PA-IIL) from *Pseudomonas aeruginosa* could help to promote their tissue infectivity and pathogenicity (Winzer *et al.*, 2000).

2.3.5 In higher fungi

In mushrooms, lectins probably play an important role in dormancy, growth and morphogenesis, morphological changes consequently on parasitic infection and molecular recognition during the early stage of mycorrhization (Guillot and Konka, 1997). The fungal lectins play different roles in different circumstances, for example, the explosive growth of sporomes is accompanied by depolymerisation of stored material and movement of sugars that is directly controlled by lectins via their specific binding sites or indirectly via an enzyme system. For glycoprotein enzymes, lectins can bind to their carbohydrate moiety to repress or activate the enzymes (Guillot and Konka, 1997). Since the amounts of lectin present in *Pleurotus cornucorpiae* increases gradually from the primordium to the mature state (Kaneko *et al.*, 1993; Oguri, 1996), the lectin may be involved in the cohesion of hyphae during the development stage of the basidiome. Moreover, it has been observed that the level of lectins in the sporomes varied with the time of harvest that may be connected with the lifting of dormancy of the mycelium. As with plant lectins, the lectins might be involved in specific recognition events between the tree and their ectomycorrhizal symbiont.

2.4 Biological activities of lectins

From their carbohydrate specificity, lectins can agglutinate cells, for example, erythrocytes resulting in hemagglutination activities (Pusztai, 1991). Lectins have been

implicated in various biological activities as a result of their recognition of carbohydrates (Lis and Sharon, 1998).

2.4.1 Cell agglutination

Each lectin molecule, which generally contains two or more carbohydrate binding sites, can interact with cells by combining to sugars on their surface, thus, cross-linking the cells and resulting in the phenomena of cell agglutination and their subsequent precipitation (Figure 3) (Sharon and Lis, 1989). So the red blood cell agglutination or hemagglutination of lectins is the major attribute of these proteins, and used routinely for their detection and characterization. Lectins also form cross-links between polysaccharides or glycoprotein molecules in solution, and induce their precipitation reaction (Pusztai, 1991).

Agglutination is a complex process that is effected by several internal factors such as molecular properties of lectin (e.g. molecular size, number of carbohydrate binding sites, binding affinity, cell surface properties, number and accessibility of lectin to binding sites, metabolic state of cell (Wood, 1995). Additionally, conditions occur during agglutination process, in particular, temperature, type of cell and lectin used, and cell concentration, may also be considered as external parameter effects for the agglutination. The agglutination and precipitation activities of lectin are similar to antibodies which can be inhibited by low molecular weight compound called haptens. In this case sugars perform as hapten for inhibition assay for indication of carbohydrate structure that the lectin is specific for, and are present on the cell surface (Sharon and Lis, 1989) (Figure 3).

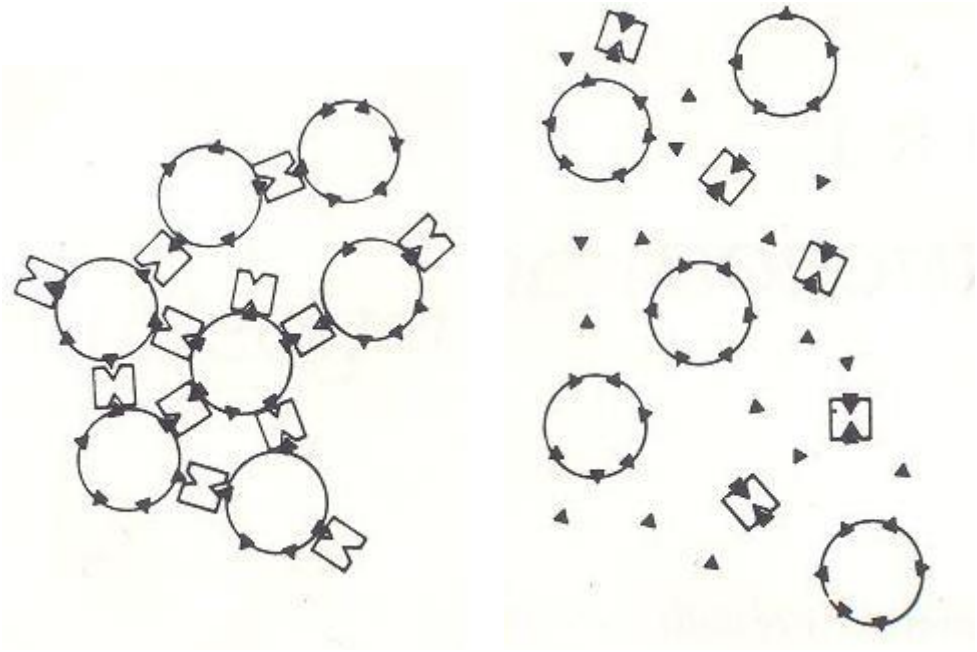

Figure 3. Schematic representation of cell agglutination by a lectin and of hapten inhibition of the agglutination.

Source: Sharon and Lis (1989)

2.4.2 Carbohydrate specificity

On the basis of their specificity, lectins are classified into 5 specificity groups according to the monosaccharide that they exhibit the highest affinity with; galactose/N-acetylgalactosamine, N-acetylglucosamine, N-acetylneuraminic acid, fucose, and mannose. Relevant for the biological activities of the lectins is the fact that of the numerous monosaccharides found in nature, only those listed above are typical constituents of surfaces of eukaryotic cells (Figure 4) (Lis and Sharon, 1998). The binding affinities of lectins are much lower than enzyme and it is also dynamic and reversible. The binding constant of lectin-monosugars interactions is relatively low (in the mM range) and lower than lectin-oligosaccharide interactions (Wood, 1995). This is due to the

oligosaccharides interacting stronger with various secondary binding sites. Throughout nature, galactose and N-acetylgalactosamine specific lectins appear to be the most abundance, followed by mannose specific lectin (Lis and Sharon, 1998). In recent years, numerous oligosaccharides and glycoproteins of known structures have been widely employed for defining the specificity of lectins that interact poorly or not at all with any monosaccharide.

2.4.3 Antiviral activity

Wood *et al.* (1999) proposed that monocot mannose-binding lectins, e.g. Liliaceae, Amyryllidaceae, and Orchidaceae lectins, exhibit anti-retroviral activity. That is possible for applications in crop protection field. *Pleurotus ostreatus* lectin (POL) has also been tested for its ability to inhibit HIV-1 reverse transcriptase. No antiviral activity has been displayed but instead POL exerted a powerful antitumour activity (Wang *et al.*, 2000).

2.4.4 Antibacterial activity

Owing to the physiological functions of plant lectins in nature, antibacterial activity of lectins has been intensively discussed (Peumans and Van Damme, 1995). However, only a few researches on antibacterial lectins have been published. Antibacterial activity of cell wall lectins from potato has been reported, and it was found that the lectins can immobilized only avirulent strains of *Pseudomonas solanacearum*. However, they cannot recognize virulent strains at all (Gozia *et al.*, 1993). Another study has been conducted with the apple (*Datura stramonium*) lectins (Broekaert and Peumans, 1986). The lectins mediated blocking of bacterial motility *in vitro*.

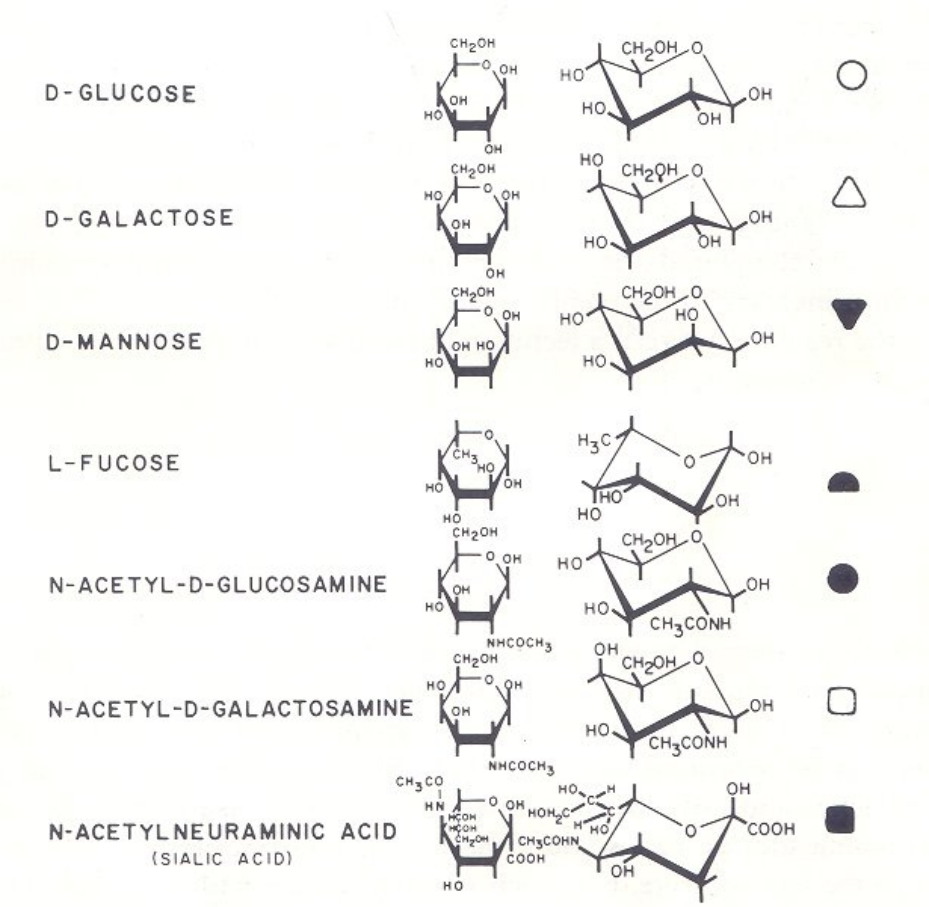

Figure 4. Structures of monosaccharides on cell surface of microorganisms, which are specific for lectin interaction.

Source: Sharon and Lis (1989)

2.4.5 Antifungal activity

Many studies of plant lectins have assumed that they are implicated in host defense mechanism as antifungal proteins. However, to date only a small number of lectins have been reported to have antifungal activity. The antifungal properties of plant lectins have been found from potato tuber (Gozia *et al.*, 1993), *Amaranthus caudatus* seed (Verheyden *et al.*, 1995), stinging nettle rhizomes (Broekaert *et al.*, 1998), wheat germ (Ciopraga *et al.*, 1999), and recently from *Phaseolus vulgaris* seed (Yu *et al.*, 2001). Some of them are chitin binding lectins. The most potent antifungal lectin belongs to class I chitinase (Collinge *et al.*, 1993). Eventually, the antifungal activity assay was carried out in the higher fungi; *Agrocybe cylindracea*, *Agaricus bisporus* (Wang *et al.*, 2001) and *Lyophyllum shimeiji* lectins (Lam and Ng, 2001). Manifest antifungal activities have not been demonstrated. Thus the discovery of antifungal lectins from plants and also higher fungi are of interest.

2.4.6 Mitogenic stimulation of lymphocytes

Previous works have demonstrated that some mushroom lectins not only regulate cell growth and morphological changes but also activate macrophages and lymphocytes (Ngai and Ng, 2004). T-cell activation and proliferation are initiated by binding ligands to T-cell receptors, subsequently activating the signaling cascades: tyrosine phosphorylation of membrane bound *lck* and *fyn* kinases, intracellular calcium mobilization, IL-2 gene expression and then lymphocyte proliferation (Imai, 1998; Ho *et al.*, 2004). Some mitogenic activities of mushroom lectins have been reported from *Volvariella volvacea*, *Boletus satanas* Lenz, *Flammulina velutipes*, *Ganoderma lucidum*, *Lentinus edodes*, and *Agrocybe cylindracea*. A recent study demonstrated that *Volvariella volvavea* lectin possessed strong mitogenic activities towards lymphocytes which were

10-fold more effective than Concanavalin A (Ho *et al.*, 2004).

2.4.7 Antiproliferative activity and cytotoxicity

Lectins, in particular mushroom lectins, have recently been shown to be of great interest since they have been reported as potential anticancer reagents that can seek out and stop multiplication of cancer cells (Kenyon, 2001). For example, *Agaricus bisporus* lectin shows antiproliferation activities against human colon cancer cell lines HT29 and breast cancer cell lines MCF-7. The Galβ1-3GalNAc∝ (Thomsen-Friedenreich: TF antigen)-binding lectin (ABL) elicits a pronounced dose-dependent decline of ^{3}H-thymidine incorporation to these cancer cells with maximal effects of 87% and 50% for HT29 and MCF-7 respectively at 25 μg/ml in serum free medium (Wang *et al.*, 1998b; Yu *et al.*, 1999; 2001).

Wang *et al.* (1998b) demonstrated that *Volvariella volvacea* lectin exhibited its antitumor activity against Sarcomo S-180 cells. It can retard the growth of tumor cells by the prolongation of life span of mice by 63 to 100%. Beside that the lectin shows the potential immunomodulatory activity by enhancing the transcriptional expression of interleukin-2 and interleukin-γ, indicating that it exerts its immunomodulatory effects via the cytokine regulation (She *et al.*, 1998).

The lectin from *Grifola frondosa* is reported to be cytotoxic against HeLa cells (Guillot and Konska, 1997). The minimum concentration that could induce all cells death is required at 25 μg/ml. Two *Tricholoma mongolicum* lectins, TML-1 and TML-2, could also stimulate the production of nitrite ions by macrophage, and activate the macrophage in mice as well as inhibit the growth of Sarcoma 180 cells (Wang *et al.*, 1995).

2.5 Potential applications of lectins

Lectins are applied predominantly according to their carbohydrate recognition which is based on precipitation and agglutination reaction (Lis and Sharon, 1998). They have been widely applied in scientific research particularly in clinical, biochemical, and biotechnology studies (Gilboa-Garber *et al.*, 1997). Several of them have been commercially utilized (Wang *et al.*, 1998b).

2.5.1 Biocontrolling agents

Since some monocot mannose-binding lectins display the potent host defense activity to bacteria that attack plants and even to insects, it is possible for the application of these plant lectins in crop protection against insects and nematodes (Wright *et al.*, 1999b). Additionally, monocot lectins from bulbs often exhibit anti-retroviral activity that might be investigated as human immunodeficiency virus (HIV) drugs (Wood *et al.*, 1999).

2.5.2 Lectin derivatives preparation

For numerous purposes, lectin derivatives are required. Lectins labeled with enzymes, biotin-avidin or even radioactive compound, has been produced, and their interaction with cells and macromolecules may be followed by the immunological techniques (microscopic, ELISA, or cell sorting by FACS). For example, *Aleuria aurantia* lectin (AAL) was used in an ELISA for analysis of fucosylation of serum cholinesterase in liver disease (Kondo *et al.*, 1995). Previously, the lectin has been used for analysis of a tumor marker (Cook *et al.*, 1999).

In the past few years, lectins derivatized with fluorescence dyes, gold particles, or enzyme, have been well-known and were employed as histochemical and cytochemical agents for the detection of glycoconjugates in tissue sections, on cells and subcellulate organelles. Furthermore, they are used for studying intracellular pathways of protein

glycosylation. Additionally, lectin binding has been used to demonstrate that membrane receptors for hormones, growth factors, neurotransmitters and toxins, are glycoconjugated (Lis and Sharon, 1998).

2.5.3 Bone marrow transplantation

Some lectins have been used in organ transplantation applications. Selective agglutination by soybean agglutinin (SBA) permits separation of B, and T, mouse splenocytes. The main application of this lectin is for purging human bone marrow for transplantation. It is employed routinely for transplantation into children born with severe combined immune deficiency ("bubble children", since they are highly susceptible to microbial infections and have to be kept in a plastic bubble all the time). SBA purging is also used experimentally in bone marrow transplantation of leukemic patients, as an alternative agent to other accepted techniques for T-cell depletion, such as monoclonal antibodies (Lis and Sharon, 1998).

2.5.4 Blood group typing

Lectins are traditionally use in blood typing. The fucose-specific lectins from *Lotus tetragonolobus* and *Ulex europaeus* are employed to identify blood type O cells (Lis and Sharon, 1998). The lectin from *Dolichos biflorus* is used to distinguish between A_1 and A_2 subgroups. While the snail lectins, *Helix pomatia* and *H. hortensis* were proved to be the excellent anti-A agents, and have been used successfully in blood banks for routine typing (Kilpatrick, 2000). Moreover, peanut lectin (PNA) which is specific for Gla(ß1-3)GalNAc, is employed in the detection of "polyagglutination" which is a condition accompanying certain bacterial and viral infections, in which human red blood cells become agglutinable by antibodies normally present in the sera of nearly all adults.

If the diagnosis was not investigated in time, it may lead to serious complications and death.

2.5.5 Mitogenic stimulation

Certain lectins are potent mitogens, activating lymphocytes and inducing them for cell division; PHA and concanavalin A, for example, stimulate T lymphocytes (T-cells), while pokeweed mitogen (PWM) stimulates both T- and B- cells. The mitogenic lectins are polyclonal activators, in that they activate lymphocyte irrespective of their antigenic specificity. Prior to the advent of monoclonal antibodies to cell surface antigens, lectins were the major tools for studies of the mechanism of cell activation. Mitogenic stimulation by lectins provides an easy means to assess the immunocompetence of patients suffering from diversity of diseases, including AIDS, and to monitor the effects of various immunosuppressive and immunotherapeutic manipulations (Lis and Sharon, 1998).

2.5.6 Cancer diagnosis

In 1963, Aubb, Burger and others discovered that a plant lectin, wheat germ agglutinin, has selectively agglutinating property to murine tumour cells. It has been revealed that neoplastic cells are differing from normal cells at the glycoconjugates on the cell surface (Kilpatrick, 2000). For example, diagnosis of cancer by mitogenic index, *in vitro* selection of antitumor cell cytotoxicity, *in vivo* reduction of cancer, efficient vaccines and immunomodulation against bacterial infection (Gilboa-Garber *et al.*, 1997; Doyle and Slifkin, 1994). Beside that the endogenous lectins from animal have been used as drug or drug targets. These lectins include CD33, which were targeted for *in vivo* leukemic blast reduction; CD44, for both differentiation therapy in acute myeloid

leukaemia and as a selective drug delivery system (Kilpatrick, 2000).

2.5.7 Pharmaceutical products

Carbohydrate biochemistry on lectins has taken on a new, revolutionary excitement, accompanied by research supports in pharmaceutical companies exceeding more than a billion dollars since 1988 (Laine, 1997). Much of this has been spent on selectin systems due to the huge market for anti-inflammatory drugs. Several small start-up pharmaceutical companies devote their efforts to this field, such as Cytel (San Diego, CA), Glycomed/Alberta Research Council (Alameda, CA), GlycoTech (Rockville, MD), and Alpha-Beta (Boston, MA).

Lectins with well-defined carbohydrate specificities are now available commercially either as free or immobilized proteins on Sepharose for the purification and isolation by affinity chromatography of glycoproteins, glycopeptides, and oligosaccharides, for example; concanavalin A, lentil lectin, pea lectin, *Phaseolus vulgaris*, *Griffonia simplicifolis*, *Ricinus communis*, and *Maackia amurensis* lectins (Cummings, 1997).

2.6 Molecular structure study of mushroom lectins

Lectins are a heterogeneous group of oligomeric protein that varies widely in size, structure, molecular organization, as well as in the constitution of their binding sites (Lis and Sharon, 1998). Depending on the carbohydrate binding specificity of lectins, affinity chromatography can be effectively used for their purification (Doyle and Slifkin, 1994).

2.6.1 Molecular weight and subunits

The molecular weight of mushroom lectins can vary over the range from 13 to

190 kDa (Table 2). Most lectin molecules are comprised of two or four subunits that may or may not be identical (Lis and Sharon, 1998; Guillot and Konska, 1997). Di-, tri- or tetra- meric lectins from mushrooms with identical subunits have been reported in *Amanita pantherina*, *Agaricus blazai*, *Lactarius deterrimus*, *Ischoderma resinosum*, and *Hygrophorus hypothejus* (Wang *et al.*, 1998b; Veau *et al.*, 1999). Lectins of *Agaricus edulis, Flammulina veltipes*, and *Hericium erinaceum* are composed of non-identical subunits (Wang *et al.*, 1998b).

2.6.2 Carbohydrate moiety

In general, glycoproteins vary in carbohydrate contents, from less than 1% up to over 99% (Sharon and Lis, 1997). The carbohydrate proportion of mushroom lectins is normally ranging between 0-18% (Wang *et al.*, 1998b). No carbohydrate moiety has been found in mushroom lectins of *Aleuria aurantia, Laccaria amethystea, L. deliciosus, L. deterrimus, L. salmonicolor,* and *Flammulina velutipes* (Guillot and Konska, 1997). Some information of lectin properties isolated from mushrooms has been summarized in Table 2.

2.6.3 Isolectins

Mostly lectin sources contain not just a single lectin but usually many lectins from the same source are often very similar with only slightly differences in molecular weight, carbohydrate specificity, some physical property such as electric charge, and even the three dimension structure. A group of such lectins from the same source are termed isolectins and their origin can be explained genetically. For example, lectins isolated from mushroom, *Agaricus bisporus*, contain four isolectins (Guillot and Konska, 1997). Study of these isolectins is of interest to understand why there are so many closely related

proteins from the same source. However, it is quite difficult to separate and purify these lectins because they have essentially the same molecular weight and sugar specificity. In order to overcome this problem, ion exchange chromatography and affinity chromatography columns have been widely investigated. Moreover, a molecular biological technique as molecular cloning and expression of this protein might be performed as a powerful method.

2.6.4 Amino acid composition

Amino acid compositions have been analyzed from several mushroom lectins: *Agaricus bisporus*, *Aleuria aurantia*, *Boletus satanas*, *Fomes fomentarious*, *Grifola frondasa*, and *Lactarius deliciosus* (Guillot and Konska, 1997). *Lactarius deliciosus* lectin (LDL) contains plenty of glycine (Guillot *et al.*, 1991). *Grifola frondasa* lectins are rich in acidic and hydroxyl amino acids, glycine and alanine, but low in methionine, phenylalanine, isoleucine, and histidine (Kawagishi, 1996)

2.6.5 N-terminal amino acid sequences

Sequence similarities of known lectins provide a guideline for the detection and identification of new ones. Amino acid sequences of lectins have been increasingly elucidated. Sequences of lectins from the same or similar families are highly homologous. Thus it can be either used to group them into distinct families or to look for the evolutionary relationships between each source (Lis and Sharon, 1989). In addition, amino acid sequences of a lectin ultimately determine their overall three dimension structure and the binding site of the lectins. Examples of amino acid sequences comparison of some mushroom lectins and also immunomodulatory proteins (FIPs) extracted from mushroom are shown in Figure 5.

Table 2. Sources and some properties of lectins isolated from mushrooms.

Molecular structure	Mushroom species	Lectin	MW (kDa)	Sugar (%)	Inhibitory sugars	References
Monomeric	*Auricularia polytricha*		23	3.5	Lac, Gal	Yagi and Tadera (1988)
	Boletus satanas		63		Gal	Kretz *et al.* (1989)
	Coprinus cinereus		16.4, 16.7	0	Galß	Cooper *et al.* (1997)
	Grifola frondasa	GFL	30-52	3.3	GalNAc	Kawagishi *et al.* (1990)
	Laccaria amethystea	LAF	16	0	L-Fuc	Guillot *et al*. (1983)
	Laccaria amethystea	LAL	17	0	Lac, GalNAc	Guillot *et al.* (1983)
	Psathyrella lacrymabunda	PVL	40	0.5	GlcNAc	Kochibe and Matta (1989)
	Psathyrella velutina		40	0.5-0.7	GlcNAc	Kochibe and Matta (1989)
	Xerocomus chrysenteron		17-22	3.5		Sychrova *et al.* (1985)
Dimeric	*Agaricus edulis* II		32	2		Eifler and Ziska (1980)
	Agrocybe aegerita		44		Gal	Ticha *et al.* (1985)
	Aleuria aurantia	AAA, AAL	72	0	L-Fuc	Kochibe and Furukawa (1980)
	Amanita pantherina		43	4.3		Zhuang *et al.* (1996)
	Flammulina velutipes	FVA	22	0	-	Yathogo *et al.* (1988)
	Ischnoderma resinosum	IRA	32	4	Lac, Gal	Kawagishi and Mizuno (1988)
	Lactarius deterrimus	LdetL	36	0	D-Galß1-3D-GalNAc	Giollant *et al.* (1993)
	Lactarius salmonicolor	LSL	37	0	D-Galß1-3D-GalNAc	Giollant (1991)
	Paxillus atrotomentosus		40	0		Haiselova *et al.* (1995)
	Pleurotus cornucopiae	PCL-a	32			Yoshida *et al.* (1994)
	Pleurotus ostreatus		80-87	14	GalNAc	Conrad and Rudiger (1994)

Table 2. (Continued)

Molecular structure	Mushroom species	Lectin	MW (kDa)	Sugar (%)	Inhibitory sugars	References
Dimeric	*Lactarius deliciosus*	LDL	37	0	D-Galß1-3D-GalNAc	Guillot *et al.* (1991)
	Marasmius oreades		50	5.4		Horejsi and Kocourek (1978)
	Pleurotus cornucopiae	PCL-b	31			Yoshida *et al.* (1994)
	Volvariella volvacea	VAG	26			Lin and Chou (1984)
	Tricholoma mongolicum	TML-1, TML-2	37		Lac	Wang *et al.* (1995)
Trimeric	*Fomes fomrntarius*		60	25	GalNAc, Raf	Horejsi and Kocourek (1978)
	Agaricus bisporus	ABA IV	64	7.7		Sueyoshi *et al.* (1985)
		ABA II	64	3.7		
		ABA III	64	2		
	Agaricus blazei		60-70	11	-	Kawagishi *et al.* (1988)
	Agaricus edulis I		60	18		Eifler and Ziska (1980)
	Lactarius lignyotus		98	4		Sychrova *et al.* (1985)
	Phallus impudicus		75	13.9		Entlicher *et al.* (1985)
	Agaricus campestris	ACH	64	4	-	Sage and Connett (1969)
	Clitocybe nebularis		70	2.6	Lac, GalNAc	Horejsi and Kocourek (1978)
	Hericium erinaceus	HEL	54	1.5	NGNA	Kawagishi *et al.* (1994)
	Laetiporus sulphureus	PSL	190	0	D-LacNAc	Konska *et al.* (1994)
Polymeric	*Pholiota aurivella*	PAA	100	4.5		Kawagishi *et al.* (1991)
	Psilocybe barrerae	PBL	75	9.5		Hernandez *et al.* (1993)

Source: Wang *et al*. (1998b) and Guillot and Konska (1997)

AAL	PTEFLYTSKI	AAISWAATGG	RQQRVYFQDL	NGKIREAQRG	GDNPWTGGSS	50
ABL	TYTISIRV	YQTTPKGFFR	PVERTNWKYA	NGGTWDEVRG	EY-VLTMGGS	47
APL	IFAVGETQGE					
PAL	YSVTTPNSVK	GGTNQG				
Cgl-I	MLYHLFVNNQ	IKLQDDFKAE	AVATIRSSVF	NSKGGTTVFN	FLSAGENILL	50
Cgl-II	MLYHLFVNNQ	VKLQNDFKPE	SVAAIRSSAF	NSKGGTTVFN	FLSAGENILL	50
FIP-vvo	STDLTQLLFF	IAYNLQKVNF	DYTPQWQRGN	PSSYIDAVVF	PRVLTNKAYQ	50
FIP-fve	SA TSLTFQ	LAYLVKKIDE	DYTPNWGRGT	PSSYIDNLTF	PKVLTDKKYS	48
LZ-8	SD--TALIFR	LAWDVKKLSF	DYTPNWGRGN	PNNFIDTVTF	PKVLTDKAYT	48
AAL	QNVIGEAKLF	SPLAAVTWKS	AQGIQIRVYC	VNKDNILSEF	VYDGSKWITG	100
ABL	GTSGSLRFVS	SDTDEIFVAT	FGVHNYKRWC	DIVTNLTNEQ	TALVINQEYY	97
Cgl-I	HISIRPGENA	IVFNSRTKGG	AWGPEERVPY	AGKFKGPNPS	ITVLDHGDRF	100
Cgl-II	HISIRPGENV	IVFNSRLKNG	AWGPEERIPY	AEKFRPPNPS	ITVLDHGDKF	100
FIP-vvo	YRVVTGDKDL	GIKPSYSVQA	DGSQKVNLLE	YNGGYGVADT	TTIKIYVVDP	100
FIP-fve	YRVVVNGSDL	GVESNFAVTP	SGGQTINFLO	YNKGYGVADT	KTIQVFVVIP	98
LZ-8	YRVAVSGRNL	GVKPSYAVES	DGSQKVNFLE	YNSGYGIADT	NTIQVFVVDP	98
AAL	QLGSVGVKVG	SNSKLAALQW	GGSGSAPPNI	RVYYQKSQGS	GSSIHEYVWS	150
ABL	GVPIRDQARE	NQLTSYNVAN	AKGRRFATEY	TVTEGIISRP	ISSSDKCFIR	147
Cgl-I	QILFDNATAI	YYTKRIKENA	AAIAYSAENS	LFSSPVTVDI	HGLLPPLPPA	150
Cgl-II	QIRFDYGTSI	YYNKRIKENA	TAIAYNAESS	LFSSPVTVDF	HGSLPALPPA	150
FIP-vvo	SNGNQYLI	AQWK				112
FIP-fve	DTGNSEEYII	AEWKKT				114
LZ-8	DTNNDFII	AQWN				110
AAL	GKWTAGASFG	STVPGTGIGA	TAIGPGRLRI	YYQATDNKIR		312
ABL	LPSQKS					153

Lectin source: AAL=*Aleuria aurantia*; ABL= *Agaricus bisporus*; APL= *Amanita pantherina*; Cgl-I and Cgl-II= I and II from *Coprinus cinereus*; PAL= *Pholiota aurivella*; LZ-8= immunomodulatory protein from *Ganoderma lucidum*; FIP-fve and FIP-vvo= fungal immunomodulatory proteins from *Flammulina velutipes* and *Volvariella volvacea* respectively.

Figure 5. Amino acid sequence comparison of some mushroom lectins and immunomodulatory proteins (FIPs).

Source: Wang *et al.* (1998b)

2.6.6 Three-dimension structure analysis of lectins

Lectins are employed in a number of applications in immunological and biochemical fields. They can be regarded as model systems for studying the molecular basis of protein-carbohydrate interaction. The structures of many plant lectins, including some lectin-carbohydrate complexes, are known at high resolution. (Hirabayashi, 1997a). More than 80 crystal structures of lectins isolated from 15 different plants particularly legume lectins have been elucidated (Rini, 1995; Imberty *et al*., 2000). The three dimension structures of a number of animal lectins have also recently been reported. However, the specific mechanisms by which lectins can discriminate between different but closely related oligosaccharides are not well understood. In order to gain a detailed understanding of the mechanisms which govern their exquisite carbohydrate-binding specificity, further knowledge of their high-resolution three-dimensional structures and conformation are required.

Due to the importance of their biological role, there is increasing interest in fungal lectins. Several fungal lectins including the lectins from *Flammulina velutipes* (Hirano *et al*., 1987), *Aleuria aurantia* (Nagata *et al*., 1991; Fujihashi *et al*., 2003), *Pleurotus ostreatus* (Chattopadhyay *et al*., 1999), *Pleurotus cornicopiae* (Oguri *et al*., 1994), and *Sclerotium rolfsii* (Leonidas *et al*., 2003) have been successfully crystallized. Also, partial structural characterization of two lectins from *Coprinus cinereus* (Cooper *et al*.,1997) and *Rhizoctonia solani* (Candy *et al*., 2001) has been also reported based on their primary sequences; to date, only the crystal structure of *Aleuria aurantia* has been determined (Wimmerova *et al*., 2003).

Crystals of *Aleuria aurantia* and *Pleurotus ostreatus* lectins showed hexagonal bipyramid in shape (as shown in Figures 6 and 7 respectively) whereas the

crystals of *Sclerotium rolfsii* lectin are needle-like crystal (Figure 8). The crystal structure of *Aleuria aurantia* lectin (AAL) complexed with fucose has revealed that each monomer consists of six-bladed ß-propeller fold and of a small antiparallel two-stranded ß-sheet that plays a role in dimerization (Figure 9).

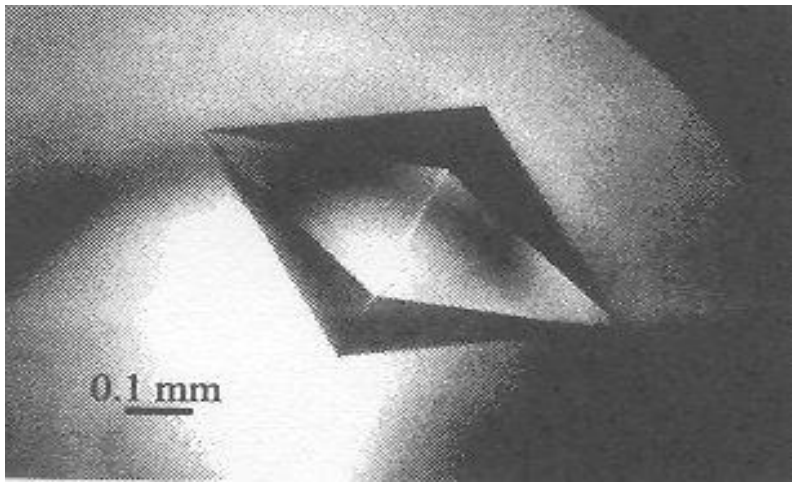

Figure 6. The crystal of lectin from *Aleuria aurantia*.

Source: Fujihashi *et al*. (2002)

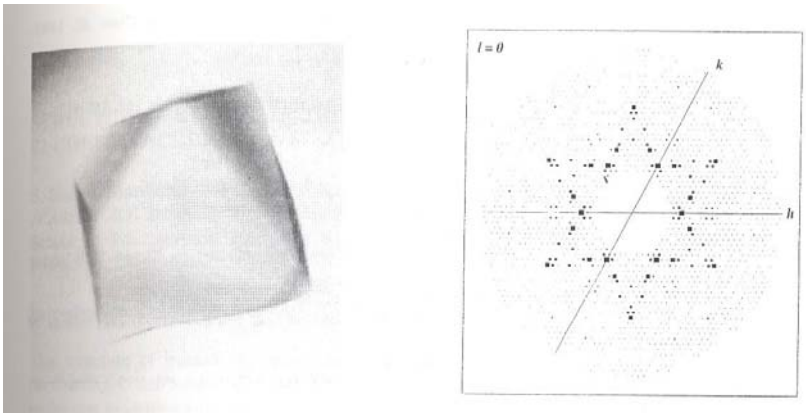

Figure 7. Crystal and X-ray diffraction pattern of lectin from *Pleurotus ostreatus*.

Source: Chattopadhyay *et al.* (1996)

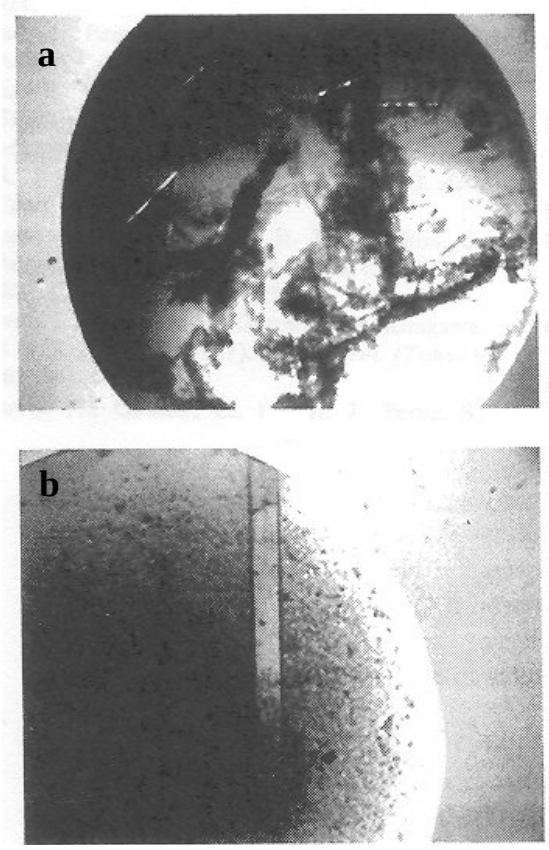

Figure 8. Photograph of the *Sclerotium rolfsii* lectin (SRL) crystals, which were grown from the initial screening (a) and after optimization of the crystallization conditions (b).

Source: Leonidas *et al*. (2003)

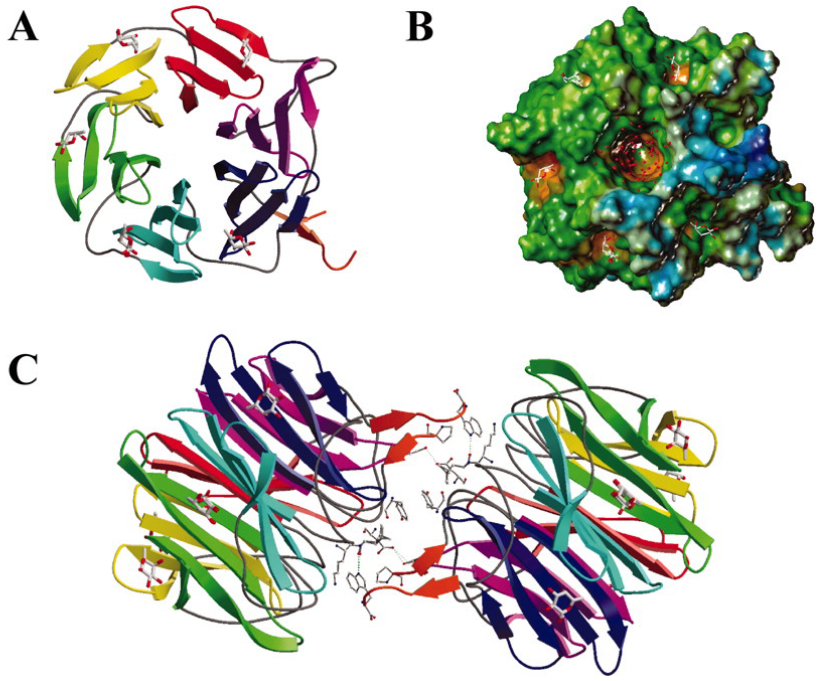

Figure 9. Ribbon diagram of *Aleuria aurantia* lectin (AAL) monomer complexed with fucose (A), Connolly surface of AAL color-coded according to the hydrophobicity potential (B), and dimer of AAL with stick representation of the amino acids involved in the interaction of monomers (C).

Source: Wimmerova *et al.* (2003)

2.7 Tropical mushrooms as a valuable source of lectins

Mushrooms are macrofungi belonging to Subdivision Basidiomycota that produce conspicuous fruiting bodies (Alexopoulos *et al*., 1996). They are diverse in their forms, varying from the feature of cap and stem. Several mushrooms form beneficial structures to plants as mycorrhizae (Nilsson and Presson, 1978a; 1978b). Others degrade dead organic matters, or act as parasites on living organisms (Arora, 1986). Through their ability in breaking down the dead materials, mushrooms play the essential role in the ecological balance of the environment (Læssøe, 1998). To grow and form fruiting bodies, most of them are confined to a very specific environment. Their habitats and climate including their indigenous plants are major factors that indicate their diversity (Nilson and Presson, 1978a; 1978b; Læssøe, 1998).

The diversity of macrofungi in Thailand is extremely high, and might be the highest diversity in Asia because of the natural geography of the country. Since the distance of the country from the Northsouth is long (about 1,860 km), the broad geographic characteristics are represented (Thaithatgoon, 1998). In the North and Northeast Thailand, the diversity of mushrooms at Doi Suthep National Forest and Doi Inthanon National Forest, and Khao Yai National Park respectively has preciously been investigated (Walting, 1998). The macrofungi belonging to *Inocybe* spp., *Boletus ornatipes, B. emodensis, Pulveroboletus ravanelii, Lactarius volemus, Hiemiella retispora, Boletellus emodensis, Gyroporus castaneus-heterosporus, Strobilomyces velutipes, S. mollis, Clavulinopsis amoena, Lentaria surculus,* and *Ramaria* spp. have been found at Doi Inthanon. *Tricholoma giganteum* (*Macrocybe*), *Termitomyces aurantiacus,* and *T. clypeatus* are apparently found at Doi Suthep. Some new species, e.g. *Boletus coccineinanus* and *Rubinoboletus ballouii*, have been found at Khao Yai

and Doi Suthep (Walting, 1998).

Rodtong *et al*. (1998) have intensively studied the diversity of mushrooms at Nong-rawieng Plant Genetics Forest and Tup Lan National Park in the Northeastern Thailand. At Nong-rawieng Plant Genetics Forest composed of dry dipterocarp and mixed deciduous forests, diverse genera of macrofungi occur; for example, *Boletus, Cantharellus, Ganoderma, Hypoxylon, Lactarius, Lycoperdon, Macrolepiota, Maramius, Mycena, Pisolithus, Russula, Schizophyllum,* and *Termitomyces* have been found. But in the dry dipterocarp forest of Tup Lan National Park, Nakhon Ratchasima, six major genera: *Amanita, Boletus, Cantharellus, Lactarius, Russula,* and *Schizophyllum,* have been described. In the family *Russulaceae*, especially members of genera *Russula* and *Lactarius*, makes up the greatest number of ectomycorrhizal basidiomycetes (Rodtong and Teaumroong, 2000).

In Eastern Thailand, the diversity of macrofungi has been reported in two tropical areas, Khao Kitcha Koot National Park and Khao Sai Dao wildlife sanctuary (Klingesorn *et al*., 1998a, 1998b). From the total of 79 genera of 35 families found, the dominate species found in Khao Kitcha Koot National Park are *Alboleptonia sericella*, *Earliella scabrosa*, *Fomitopsis rhodophaea*, *Lycoperdon peratum*, *Marasmius candidus, Microporus xanthopus, Perenniporia tephropora, Phellinus griseoporus, Polyporus grammocephalus, Polyporus tenniculus, Ganoderma australe, Hexagonia tenuis*, *Pycnoporus sanguineus*, *Stereum ostrea*, *Termitomyces microcarpus, Trametes elegans*, and *Trametes menziezii*. Whereas only 63 genera of 29 families have been found at Khao Sai Dao wildlife sanctuary, and the prominent species were *Cookeina* sp. nov., *Cookeina sulcipes, Cyathus striatus*, *Earliella scabrosa*, *Filoboletus manipularis*, *Polyporus grammocephalus*, *Polyporus*

squamosus, *Stereum ostrea*, *Thelephora* sp. nov., *Tremella fuciformis*, and *Tremetes incana*.

The great diversity of mushrooms of Thailand has led to their increased consideration as alternative sources of lectins. This study aims to investigate mushroom lectins from tropical forests of the country. Data on structures and functions of lectins would be beneficial to their applications and structural based-drug design in the future.

CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals and reagents

Chemicals and reagents used for the extraction, purification, characterization, and the manual crystallization (hanging drop technique) of mushroom lectins were of analytical grade. Ammonium peroxodisulphate (APS), bromophenol blue, bis-N, N''-methylenebisacrylamide, calcium chloride 2-hydrate, Coomassie blue R-250, Ethylenediamine tetra-acetic acid (EDTA), 2-mercaptoethanol, magnesium chloride solution, glycerol, glycine, sodium azide, sodium dodecyl sulphate (SDS), Tris (hydroxymethylamine), N-Tris (hydroxymethyl) methylglycine, N, N', N'', N'''-tetramethylethylenediamine (TEMED) were purchased from BDH-Merck Chemicals LTD (Poole, U.K.) Agarose, Bradford reagent, benzamidine hydrochloride (98%), mucin type III partially purified from porcine stomach, sodium metabisulphite, and periodic acid-Schiff (PAS) kit were products of Sigma-Aldrich Chemical Company (St. Louis, MO, U.S.A.). Cyanogen-bromide-activated Sepharose 4B was product of Amersham Pharmacia Biotech (Uppsala, Sweden). Amicon filters were purchased from Millipore Corporation (Bedford, U.S.A.). Other chemicals were purchased from a variety of suppliers. The microbiological media used for culturing of mushroom mycelium and for the antimicrobial susceptibility test; Mueller-Hinton agar (MHA), Malt extract agar (MEA), and Nutrient broth (NB), were obtained from Oxoid (Basingstoke, U.K.) For hemagglutination assay, human blood was obtained

from the blood donation office of The Thai Red Cross Society in Nakhon Ratchasima Province, Thailand. Whereas a variety of animal blood was supplied from the Division of Production and Supply, National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand. During research at Liverpool John Moores University, the rat and rabbit blood was obtained from Liverpool John Moores University Life Science Support Unit.

3.2 Instrumentation

All instruments required for the mushroom collection and the determination of lectins accumulated in mushroom species are located at the Center for Scientific and Technology Equipment at Suranaree University of Technology, Nakhon Ratchasima, Thailand. For cytotoxic activity assay, the experiment was performed at the Natural Products Research Section, Research Division, National Cancer Institute of Thailand, Bangkok, Thailand. All instruments needed for protein purification and characterization were located at the School of Biomolecular Sciences, Liverpool John Moores University, Liverpool, U.K. For amino acid sequence analysis which including N-terminal analysis and In-gel digestion, the instruments were located at the Department of Biochemistry, University of Liverpool. For X-ray diffraction, the experiment was conducted at the SRS of the Council for the Central Laboratory of the Research Councils (CCLRC), Daresbury Laboratory, Daresbury, Warrington, Cheshire, U.K.

3.3 Collection of mushroom specimens

Throughout this study, fruit bodies of mushrooms were collected during the rainy season August-October, 2002 and June-August, 2003 from a variety of natural habitats, natural forests, and local markets in the Northeastern, Central, and Western Thailand. Specimens were collected and deposited with care according to the protocol described by Arora (1986) and Bandoni *et al.* (1996). Before gathering, number of each mushroom was approximately counted and recorded. About 100 grams of fresh fruit body was appropriated for the mushroom identification and further lectin study. For field surveys, all mushroom specimens were photographed before collecting, then wrapped into a piece of wax paper to keep them fresh. Data concerning the locality, habitats, and the date of collection, were recorded. After returning to the laboratory, photographs of representative specimen from each mushroom specimen were taken, and their morphology was characterized. Spore prints were also examined for some specimens. The spore-bearing structure of some mushrooms was sectioned using a razor blade and mounted in triplicate on the microscope slide with test chemical reagents; 10% ammonium hydroxide, Melzer's reagent, and lactophenol (Appendix A), then examined under light microscope. The identification method followed Arora (1986), Soytong (1994), The Royal Academy Institute (1996), Chansrikul (1999), Bon (1987), Pegler and Spooner (1992), Nilsson and Persson (1978), and Phillips (1981). Pure culture isolation was also attempted from both spore and fruit body (Jansrikun, 2000) using malt yeast extract broth (Appendix A) for 14 days with shaking. All materials were dried by using the hot air oven (at 40°C) until moisture humidity was about 5-8%. The dried specimens were stored at low temperature (below 25ºC) until lectin determination.

3.4 Determination of accumulated lectins in mushroom specimens

3.4.1 Lectin extraction

Dried mushroom fruit bodies were ground into powders using Waring blender for 5 min at high speed. Five grams of each mushroom powder were homogenized in a cold mortar and pestle with 10 times (w/v) of 0.01 M phosphate buffer saline (PBS, pH 7.4) containing 0.02 M sodium bisulphite then left at 4°C for 2 h. Filtrates of the homogenates were collected by centrifugation at 10,000 rpm for 30 min at 4ºC, and applied for the detection of lectin accumulations using hemagglutination assay.

3.4.2 Hemagglutination assay

Since hemagglutination have been performed as the most simple method for all lectin determination, accumulations of lectins in crude extracts of these mushroom specimens were detected by testing for hemagglutination activity against human (A, B, and O blood groups) and animal (goose, rabbit, rat, and sheep) red blood cells. Blood collected from both human and animal bodies was maintained in 8% of sodium citrate. Then red blood cells were washed three times with 0.01 M PBS (pH 7.4), and resuspended in the same buffer solution to give a 5% cell suspension. The hemagglutination assay was performed at room temperature using two-fold serial dilution of mushroom filtrate (crude extract) (Wright, 1998). The crude extract (25 µl) of the lectin was transferred in triplicates into the wells of a microtiter plate (130×85×15 mm) containing 8×12 wells with U-shaped bottom and 25 µl of the red blood cell suspension was added to PBS buffer. For control, the red blood cell suspension was added to buffer. Then the plates were left at room temperature at 37ºC

for 1 h before reading the results.

Positive results were recorded when the agglutination occurred, when red blood cells formed a red carpet which covered the whole bottom of the wells of the microtiter plate. In contrast to negative results that agglutination failed to take place, and all erythrocytes were settled down to the bottom of the well. When looked at from above, a red dot can be observed. Positive and negative results were completely different from each other, it could be easily observed by comparing between negative and positive wells. However, care must be taken in order not to mistake hemolysis for agglutination. The different between these two phenomena could be simply observed if the wells are looked at from the side. In hemagglutination, the red layer covers only the bottom where as the supernatant is colorless. If hemolysis has occurred, the whole well is filled with a red and transparent solution. The agglutination was scored from 0 which is equivalent to no agglutination, to 4 when full agglutination could be observed. When the serial dilution was performed across the plate, the lowest concentration of the lectin which gave 4 scores would be calculated.

The hemagglutination titer, which is defined as the reciprocal of the highest dilution exhibiting hemagglutination, was recorded. The mushroom extract, which showed high in hemagglutinating activity, was selected for biological activity test.

3.4.3 Total protein estimation

Total protein concentration in crude extract of mushroom was determined by the method of Bradford *et al.* (1951) using bovine serum albumin as a standard. However, absorbance at 280 nm method according to Bollag *et al.* (1996) was also used to determine the protein content of the eluates from the affinity chromatography

column and to monitor elution of protein profiles during chromatography.

3.5 Selection of mushroom lectins according to their biological properties

3.5.1 Extraction procedure

In order to clarify their biological properties, more purity of the mushroom extracts must be increased thus the extraction method was modified. Dried mushroom fruit bodies were ground into powder by using a blender at high speed, pausing for several seconds between each pulse. Lectins were extracted from mushroom powder by homogenizing the powder in a cold mortar and pestle with 10 times (w/v) of 0.01 M PBS, pH 7.2 containing 1mM benzamidine, 0.1% 2-mercaptoethanol, and 1.5% (w/v) polyvinylpyrrolidone (PVPP). The homogenate was kept by stirring in cold room at 4ºC overnight. Then the homogenate was centrifuged at 14,000×g for 30 min at 4ºC. The crude extract was precipitated from homogenate by using 30% w/v of ammonium sulfate. Ammonium sulfate powder was slowly added with gentle mixing and left at 4ºC overnight. After centrifugation at 35,000 rpm for 30 min, at 4ºC, the protein was obtained from the supernatant and consequently submitted to further biological activity test.

3.5.2 Cytotoxicity test against cancer cell lines

Two types of cancer cell lines, human epidermoid carcinoma (KB) and human cervical carcinoma were investigated. These cells were trysinized before seeding at a density of 2×10^4 cells/µl in 96 well plates for 24 h, at 37ºC in an atmosphere of 5% CO_2. Then serial concentrations of the lectin extracts (100 µl/well)

were added before further incubation for 72 h (in the same as previous condition). At the end of the incubation, the medium was removed and the plate was washed with PBS. MTT (3-[5, 5-dimethylthyazol-2-yl-2,5-diphenyltetra zolium bromide) solution (100 µl/well) was then added following by further incubation for 4 h. Absorbance at 550 nm was measured using microtiter reader after incubation with dimethyl sulfoxide (100 µl/well) for 30 min. Reagents and controls were included with the absence of cells or the crude extract, respectively.

$$\text{Viability}(\%)\ \text{of tumor cells} = \frac{\text{Abs}_{550\text{nm}}\ (\text{Sample})}{\text{Abs}_{550\text{nm}}\ (\text{Control})} \times 100\%$$

3.5.3 Antimicrobial activity test

The mushroom extracts which dissolved in 10 mM Tris-HCl, pH 8 was sterilized by filtration through a 0.45 µm membrane filter. Agar disc diffusion method was then carried out according to Murray *et al.* (1999).

3.5.3.1 Antibacterial activity test

For the antibacterial assay, the test organisms used were *Bacillus cereus* ATCC 6633, *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 10145, and *Escherichia coli* ATCC 25922. For inoculum preparation, the test culture strains were transferred from nutrient agar slants into test tubes containing nutrient broth, and grown overnight by shaking at 37ºC. Then the overnight culture was diluted with sterile phosphate buffer saline (PBS), pH 7.4 to yield a 0.5 McFarland suspension (approximately 10^8 CFU/ml). The prepared inoculum was then

aseptically inoculated on Mueller Hinton Agar (MHA) agar plate by three-dimension swab technique (streaking each plate in horizontal, vertical directions and around the edge with a sterile swab). The filter paper discs (6 mm in diameter, Whatman, U.K.) were each impregnated with 50 µl of the purified lectins and placed on the inoculated agar, then incubated at 37ºC for 24 h. Each test was carried out in triplicates with controls. The filter paper disc containing the reference antibiotics (ampicillin) (10 mg/ml) was used as a positive control.

3.5.3.2 Antifungal activity test

For the antifungal activity assay, *Saccharomyces cerevisiae* ATCC 26108, *Candida albican* ATCC 10231, *Aspergillus niger* ATCC 6275, and *Penicillium digitatum* ATCC 201167, were grown in 2% (w/v) malt extract broth (MEB) for 48 h at 30ºC, then streaked on 2% (w/v) malt extract agar (MEA) for the agar diffusion experiments. The concentration of the fungal suspension tested was adjusted to 10^7 cells or spores/ml. The lectin extracts were applied as described above. One hundred units of nystatin were used as positive control. In this test, the inhibitory zones were detected after incubation for 48 h at 30ºC. All tests were also performed in triplicates.

3.5.4 Preliminary purification of some mushroom lectins

Some mushrooms were selected as representative samples for preliminary lectin purification. In these experiments, different types of affinity column; mucin-Sepharose 4B, desialylated mucin Sepharose 4B, and N-acetyl-D-galactosamine (GalNac), ion exchange chromatography (DEAE-Sapharose and CM-Sepharose) and

also gel filtration (Sephadex G75) were investigated. Then all information on lectin purification protocol of each mushroom type was recorded.

3.6 Purification and characterization of mushroom lectins

3.6.1 Extraction of mushroom lectins

Since the extraction method had been modified, it was used again for the lectin purification. Fifteen grams of the mushroom powder were homogenized with 10 times (w/v) of the ice-cold extraction buffer; 0.01 M phosphate buffer saline (PBS), pH 7.2 containing 1mM benzamidine, 0.1% 2-mercaptoethanol using a cold mortar and pestle. In order to absorb polyphenolic substances, 1.5% (w/v) insoluble polyvinylpyrrolidone (PVPP) was added after homogenization. The homogenate was extracted by stirring overnight at 4°C. The resulting suspension was filtered through cheese cloth and consequently centrifuged at 4ºC for 30 min at 14,000×g (JA14 fixed angle rotor, Avanti-J25 centrifuge, Beckman Coulter, Fullerton, CA, U.S.A.) to remove insoluble residues. Solid ammonium sulfate was next added to the supernatant to obtain 30% saturation and allowed stirring overnight in the cold room. By ultracentrifugation (45Ti rotor, L80 ultracentrifuge, Beckman Coulter, Fullerton, CA, U.S.A.) at 4ºC for 30 min at 35,000 rpm, the supernatant was collected and defined as crude extract that was ready for loading on an affinity chromatography.

3.6.2 Preparation of affinity chromatography column

In this work, three affinity chromatography columns mucin-Sepharose 4B, desialylated mucin-Sepharose 4B, and N-acetyl-D-galactosamine were investigated.

For mucin-Sepharose 4B affinity chromatography, the column material used

was cyanogen bromide activated Sepharose 4B that is a ready-to-use reactive derivative of Sepahrose 4B which is prepared by reacting cyanogens bromide with Sepharose 4B results in a reactive product to which proteins, nucleic acids or other biopolymers can be couples via primary amino groups or similar nucleophilic groups. Cyanogen bromide reacts with hydroxyl groups on Sepharose resulting in CNBr-activated Sepharose 4B with active cyanate ester group that can couple with other ligands such as protein and nucleic acids. In this study, gastric mucin is immobilized as ligand to the CNBr-activated Sepharose 4B by the following protocol.

Five grams of freeze dried S4B powder is suspended in 1 mM HCl (200 ml/g). The gel was left for swelling for 30 min and then washed for 15 min on a sintered glass filter with the same solution. A total volume of 200 ml per gram dry gel was added in several aliquots. From one gram freeze-dried powder, a gel volume of approximately 3.5 ml was obtained. Hydrochloric acid can help to preserve the activity of the reactive groups, which hydrolyze at high pH. The final aliquot of 1 mM HCl was sucked off until cracks appear in the gel cake.

In the meanwhile, the ligand solution was made up by dissolving ligand to be coupled (10 g of mucin per ml gel) in coupling buffer ($NaHCO_3$ buffer; 0.1 M, pH 8.3) containing NaCl (0.5 M). A gel: buffer ratio of 1:2 gave a suitable suspension for coupling. After the ligand solution was ready, the gel was immediately transferred to that solution. The mixture solution was gently shaking on rotary shaker overnight at 4ºC. Then the gel was transferred to blocking buffer (1 M ethanolamine or 0.2 M glycine, pH 8.0), and kept for 2 h at room temperature. The excess adsorbed protein and blocking agent were removed by washing gel with coupling and washing buffer subsequently. The gel was poured into a column and allowed to settle under gravity by

topping up the column with the buffer. When the gel had settled, a filter was placed at the top of the column, and buffer was run through the column via pump at a rate of 1 ml/min, and allowed to continue for at least 30 min. For the lectin purification, the column was equilibrated with a running buffer (Tris-HCl pH 8 containing 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM benzamidine) at volumetric flow rate of 1 ml/min for several hours until a flat base line with a non-fluctuating signal was recorded from the path monitor in absorbance at 280 nm. The column was now ready to use for the lectin purification.

For desialylated mucin-Sepharose 4B affinity column preparation, neuraminidase *Clostridium perfringens* (Sigma, St. Louis, MO, U.S.A.) 0.60 U/mg of mucin was used by dissolving in 50 ml of 0.1 M Sodium acetate buffer and incubated with 2.5 g of mucin at 37ºC for 2 days. Then ligand solution was dialyzed against coupling buffer for 6 h before transferring to the matrix solution. After the incubation at 37ºC overnight, the matrix was filtrated and loaded into the blocking buffer. The solution was left on the shaker at room temperature for 2 h before loading on the column containing the washing buffer. The column was equilibrated and kept in the cold room until used.

N-acetyl-D-galactosamine (GalNac) was prepared according to the manual using Epoxy-activated Sepharose 6B. To prepare 20 ml column, about six grams of the Epoxy-activated Sepharose 6B powder was suspended in distilled water for 1 h. Then the gel was washed with 200 ml of distilled water. Ligand solution was prepared by dissolving four grams of the ligand (N-acetyl-D-galactosamine (GalNac)) powder in the coupling buffer (50 mM sodium phosephate buffer, pH 8) before transferring to the matrix solution and loading onto the column.

3.6.3 Purification of lectins by affinity chromatography

The crude extract of lectin was slowly loaded through the mucin Sepharose 4B column (2.6×5 cm; 25 ml bed volume) by removing the buffer reservoir out. The column was run with running buffer. The P-1 peristatic pump (Pharmacia Biotech, Uppsala, Sweden) was operated at a flow rate of 0.5 ml/min and the output from the single path monitor UV-1 (Pharmacia Biotech, Uppsala, Sweden) was regularly checked. After a peak of unbound protein was observed via the Graphic 450 flat bed recorder (Lloyd Instruments Ltd., Hampshire, U.K.) and also the flat base line (the A_{280} decreased below 0.01) was reached for a moment, presumably the column was saturated with the lectins of interested, then the column was exhaustively washed with the 2 bed volumns of the same buffer. The adsorbed lectin was desorbed with the unbuffer 20 mM of 2,3 diaminopropane (DAP). Bound lectin was eluted when another peak was observed from the path monitor and shown on the chart recorder. The column was left running for another hour with the elution buffer until the peak returned to the base line. Afterwards all fractions of 6 ml were collected using a fraction collector, model 2110 (Bio-Rad, Richmond, CA, U.S.A.). The eluants were each immediately neutralized to neutral pH with 1 ml of 1 M Tris-HCl, pH 7. All 7 ml protein fractions were then checked for their absorbance by a Lambda 40 UV-VIS spectrophotometer (Perkin Elmer Instruments, Shelton, CT, U.S.A.) at 280 nm, and also hemagglutinating activity was determined in the resulting fractions, and the active fractions were identified as purified lectin. The purified lectin fractions were then pooled together, dialyzed, concentrated down by ultrafiltration, and stored in the freezer (-20°C) until further needed.

3.6.4 Dialysis of purified lectins

After each step of purification, the removal of salts or even buffer changing was needed to achieve either the efficiencies of protein binding capacity to matrix for the next step of purification or the protein purity for storage and crystallization. Therefore dialysis method that relies on the passage of molecules through a semi-permeable membrane from high concentration to low concentration region was investigated. The dialysis tubing membrane used in this study made from cellulose acetate, with a nominal molecular weight cut-off (NMWC), which will be retained by the membrane of 10 kDa. Dialysis was carried out overnight at 4ºC to minimize loss in lectin activity. At least ten times volume of changing buffer were required. Moreover, this method was frequently used for the purpose of protein concentration.

3.6.5 Concentration of protein

For crystallization, the concentration of protein in the solution had to be considerated. It should be in the range of 0.5-1 mg/ml or higher. Thus the volume of protein was decreased down by an Omega-10 ultrafiltration (Filtron, Karlstein, Germany) with polysulphone low-protein binding membrane molecular weight cut off at 10 kDa. The concentrator was operated by fitting it with a stirrer bar mechanism and a 15 microns filter, the process running by loading the protein into the concentrator and pressuring it via forcing out the dialysis buffer. As the volume decreases, the concentration of protein is increased.

3.6.6 Measurement of protein concentration by UV-spectroscopy

To ensure that the operation would succeed in each step of purification, the

samples were tested on the Lambda 40 UV-VIS spectrophotometer (Perkin Elmer Instruments, Shelton, CT, U.S.A.) using two aliquots of the appropriate buffer as blank, a standard check was carried out at wavelength of 245 to 310 nm. Then protein concentration could be calculated. According to the plant lectin, an optical density of 120 at 280 nm would correspond to approximately 1 mg/ml of protein.

3.6.7 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis (PAGE) is a rapid method for assessing the purity of proteins. In the presence of sodium dodecyl sulphate (SDS), anionic detergent which binds strongly to proteins giving its constant negative charge per unit mass, subunits of proteins are separated and molecular weights of protein can be subsequently determined. For the mushroom lectins, SDS-PAGE was carried out on a 17.5% polyacrylamide gel according to the method of Laemimli and Favre (1970) (Appendix A). Samples which were the mixture of protein sample prepared by trichloroacetic acid (TCA) precipitation (Bollag *et al.*, 1996) (Appendix B), 10 µl of upper buffer, and 10 µl of sample buffer, were heated in the presence or absence of 2-mercaptoethanol for 3-5 min at 100ºC. After electrophoresis, gels were stained with Coomassie Brilliant Blue. The molecular weight of the lectin was determined by comparing its electrophoresis mobility with the standard high molecular weight marker proteins, HMW-SDS marker kit (Amersham Pharmacia Biotech, Uppsala, Sweden). In this study, Tricine-SDS-PAGE gel electrophoresis was conducted to help resolving the smaller molecular weight lectins. The method is similar to the SDS-PAGE system but it includes tricine instead of glycine. This type of gel allows for the

resolution of small proteins between 5-20 kDa. It also reduces the effect of protein overloading on resolved proteins. The tricine gel preparation protocol is described in Appendix A.

3.6.8 Molecular mass determination by native particle gel-PAGE

The Molecular weight of the native lectin was determined using 5% polyacrylamide native particle gel in combination with SDS-PAGE. The particle gel was carried out on 5% polyacylamide gels according to Bollag *et al*. (1996). Gel was prerun at 25 mM constant current for 1 h before running at same current for another 3 h. Gel segments were cut at 5 mm and placed on stacking gel when 2nd SDS-PAGE was prepared. Then SDS-PAGE was run for two-dimensional analysis.

3.6.9 Hemagglutination test of purified lectins

A serial two-fold dilution of the mushroom lectin solution in microtiter U-plates (50 µl) was mixed with the same volume of a 2% suspension of rabbit red blood cell (RBCs) in phosphate buffer saline (PBS, pH 7.2), and incubated at 4ºC. Hemagglutination results were read after about 1 h when the negative control had fully precipitated. The hemagglutination titer, defined as the reciprocal of the highest dilution exhibiting hemagglutination, was reckoned as one hemagglutination unit. Specific activity was calculated as the number of hemagglutination unit/mg protein.

3.6.10 Sugar specificity test of lectins

The hemagglutination sugar inhibition assay was investigated for studying sugar specificity of the mushroom lectin. It was performed in a manner analogous to

the hemagglutination test. Different types of sugars; D-glucose, D-galactose, N-acetyl-D-galacosamine (GalNAc), N-acetyl-D-glucosamine, mannose, xylose, fucose, raffinose, arabinose, rhamnose, and lactose, were investigated. Serial two-fold dilutions of test sugar samples were prepared in phosphate-buffer saline PBS, pH 7.4. Each dilution was mixed with an equal volume (25 µl) of a solution of the agglutinin with 16 hemagglutination units. The mixture was allowed to stand for 30 min at 4ºC before mixing with 50 µl of 2% rabbit erythrocyte suspension. The minimum concentration of the test sugar in the final reaction mixture, which completely inhibited 16 hemagglutination units of the lectin, was calculated.

3.6.11 Heat stability and pH stability

The heat stability and pH stability of the selected lectin was examined according to the modified method of Kobayashi *et al.* (2004) and Freire *et al.* (2002). Heat stability was determined by heating aliquots of the lectin (1mg/ml lectin solution in PBS) for 30 min at various temperatures of 40, 50, 55, 65, 80, and 100ºC. Then the heated aliquots were cooled rapidly on ice, centrifuged to remove any precipitate, and assayed for hemagglutination in comparison with a control sample of lectin. Results were expressed by calculating the percentage of hemagglutination shown by the heated aliquots (titration value) compared with the control sample with representing 100%.

For the pH stability, the lectin was measured by incubating the lectin samples in the following buffers varying from pH 3-10 for 18 h at 4ºC. Different buffers were used according to pH range as follow; 50 mM glycine-HCl buffer (pH 2.0-3.0), 50 mM sodium acetate buffer (pH 4.0-5.5), 50 mM Tris-HCl buffer (pH 8.0-

8.5), and 50 mM glycine-NaOH buffer (pH 9.0-11.0). The residual hemagglutinating activity was assayed after dialysis the lectin samples against PBS for 18 h at 4ºC. Three replicates were done for each test. Results were calculated by expressing the titration values of the lectin as percentages of the titration value of the control.

3.6.12 Glycoprotein analysis using SDS-PAGE

In order to determine carbohydrate of the lectins, periodic acid Schiff (PAS) staining was investigated according to the method described by Fukuda and Kobata (1993). Briefly, the electrophoresis gel from SDS-PAGE was incubated for 1-2 h in fixation solution and following by soaking in periodic acid solution for another 1 h. The gel was rinsed in water. Meta-bisulfite solution (Appendix A) was consequently added. The gel slowly turned to yellow, and then fresh meta-bisulfite solution was added and left for 5-10 min until decolorized. The gel was then placed in Schiff's reagent and incubated until red band of carbohydrate appeared. No more staining intensity was usually seen after 2 h. The excess of reagent was removed by incubation with destaining solution (Appendix A). ß2 glycoprotein I, product of Amersham Pharmacia Biotech (Uppsala, Sweden) and chicken IgG, which was kindly obtained from Dr. Stan Lambert, were used as positive controls.

3.6.13 Amino acid sequence analysis

For the N-terminal amino acid sequence analysis, the purified lectins at the concentration of 1 mg/ml was blotted on to nitrocellulose membrane before analyzing by means of automated Edman degradation using a model 471A Protein Sequenator (Applied Biosystems, Warrington, U.K.)

In-gel digestion was carried out using an adaptation of the method of Rosenfeld *et al.* (1992). Gel slices from SDS-PAGE were cut into small pieces, placed in a microcentrifuge tube and washed (2×30 min) with the buffer containing 50% acetonitrile, 0.2 M ammonium bicarbonate pH 8.9 and then freeze-dried for 1 h. The slices were re-swollen in the buffer composing of 0.2 M ammonium bicarbonate pH 7.8, 0.02% tween 20 with 2 M urea and containing a quantity of trypsin equivalent to Ca.10% (w/w) of target protein. This buffer was added to the slices in 10-20 µl aliquots, allowing each gel slice to take up the entire buffer before adding the next aliquot. When completely re-swollen, the slices were incubated at 37ºC overnight. At the end of the incubation period, excess buffer was removed to a new microcentrifuge tube, and peptides were extracted from the gel slices with 2 lots of 60% acetonitrile, 0.1% TFA. These washes were pooled with the excess buffer, concentrated by centrifugal evaporation and applied to a PE-Biosystems PepMap C18 RP-HPLC column (100×2.1 mm) equilibrated in 0.08% TFA. Peptides were separated with a 95 min gradient of 0-64% acetonitrile in 0.08% TFA, elution was monitored at 214 nm. Suitable peptides were subject to N-terminal sequencing by Edman degradation using a model 471A Protein Sequenator (Applied Biosystems, Warrington, U.K.).

The lectin sequences were submitted to automatic alignment, which was performed by the NCBI-Blast and FASTA search system for homologous sequences.

3.6.14 Crystallization of the mushroom lectins

In order to study the three-dimension structure of the selected lectins, the purified solution of the lectins needs to be crystallized. For crystallization, molecules must be brought into a thermodynamically unstable state known as supersaturation.

When the concentration of a protein is brought above its solubility limit, the solution becomes supersaturated. At this point, the protein begins to aggregate, moving from the solution phase to a separate, insoluble phase (Bollag *et al.*, 1996) as shown in Figure 10. Aggregation occurs in two distinct stages, nucleation and growth. And three zones of saturation is divided; 1) metastable zone which nucleation rate very low, 2) nucleation zone, nucleation and growth, and 3) precipitation zone, protein does not nucleate but precipitate out of the solution.

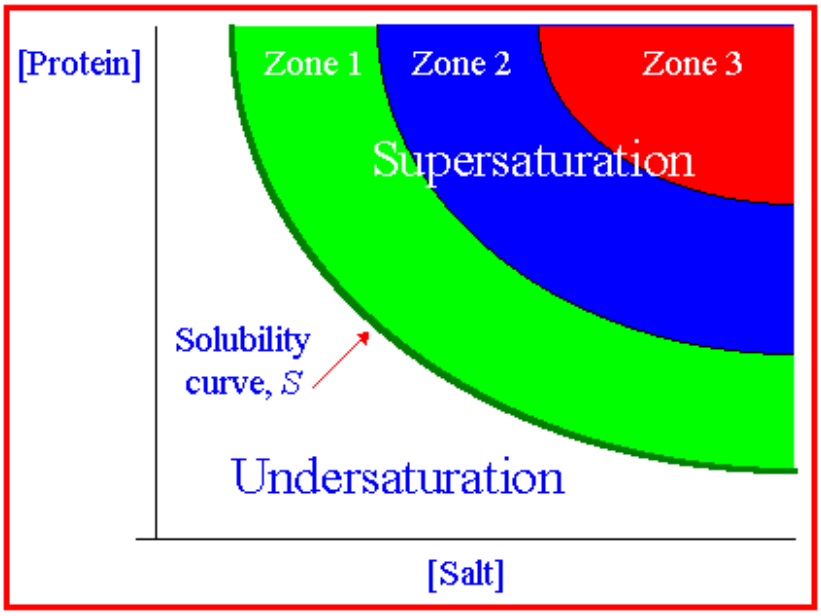

Figure 10. The solubility curve of protein which describe a phase diagram for crystallization.

Source: Howard (2002)

In this study, either hanging drop or sitting drop vapour diffusion techniques were used and different crystallization wells were set up in order to establish the optimum conditions for crystal growth (Wright *et al.*, 1999a; Sauerborn, *et al.*, 1999).

For the hanging drop technique, the experiments were performed using Linbro multi-well plate with covers and 24 flat bottom wells (1.7×1.6 cm). The buffer solution to be used as precipitation buffers was varied in pH and the amount of salt (ammonium sulphate) mixed into the buffer. A grid of 12 conditions of pH 5.5, 5.6, 5.7, and 5.8 and ammonium sulphate concentrations of 1.9, 2.0, and 2.1 M were prepared (Table 3). After a bead of high vacuum grease was applied along the top edge of the wells or reservoir (Figure 11), one ml of the prepared buffer reagent was added into reservoir.

Table 3. Sparse matrix for hanging drop technique.

pH	Concentration of ammonium sulphate salt		
	1.9 M	2.0 M	2.1 M
5.5	5.5 1.9 M	5.5 2.0 M	5.5 2.1 M
5.6	5.6 1.9 M	5.6 2.0 M	5.6 2.1 M
5.7	5.7 1.9 M	5.7 2.0 M	5.7 2.1 M
5.8	5.8 1.9 M	5.8 2.0 M	5.8 2.1 M

The procedure was repeated for the remaining buffer reagents using a clean pipette tip for each reagent to avoid reagent contamination and carry over. Crystallization droplets of 10 µl initial volume were prepared on siliconized glass cover slips by pipeting 5 µl of sample into the center of a siliconized 22 nm square cover slide and then mixed with equal volume of the buffer reagent from the reservoir. The prepared cover slide with droplet was inverted and placed over a reservoir to seal the edge of the reservoir. Crystallization was carried out at both room temperature and 4^{o}C.

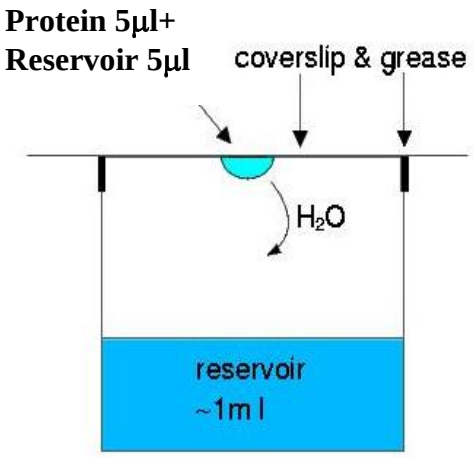

Figure 11. Hanging drop technique.

Source: www-structmed.cimr.cam.ac.uk/.../ methods.html

Lectin crystals were grown using the sitting drop technique in a series of crystallization trials using JBScreen HTS I (PEG based) and JBScreen HTS II (ammonium sulfate, MPD, Alcohol and Salt based) reagent kits (Jena Bioscience, CA). A total of 98 different crystallization wells were set up for each screening test using the crystallization robot (Appendix H) located at the Synchrotron Radiation Source at the Daresbury Laboratory, U.K. in order to establish the optimal conditions for crystal growth. A sitting drop is a mixture of 0.5 µl of lectin (10 mg/ml) and 0.5 µl of the screening reagent, and 80 µl of the same screening reagent was apply into the well as reservoirs (Figure 12). Then the crystallization plates were stored in a stable temperature environment, free of vibration at 4ºC (in the cold room). The drops were allowed to equilibrate over a reservoir of the precipitating agent for at least 3 days. The drops were examined under a stereomicroscope (Olympus VM-ILA-2 Polarizing microscope, Olympus America Inc., Melville, NY, U.S.A.) (10 to 100×magnification)

immediately after setting up the screen and once each day for the first week, then once a week after. Results were recorded by indicating whether the drop was clear, contained precipitate, and/ or crystals.

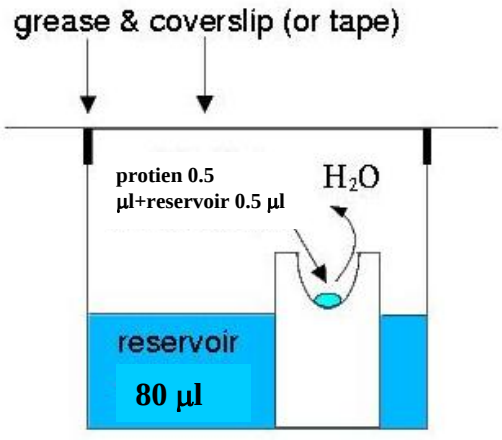

Figure 12. Sitting drop technique.

Source: www-structmed.cimr.cam.ac.uk/.../ methods.html

3.6.15 Preliminary analysis of the mushroom lectin structure

X-ray crystallography is currently the most powerful technique for structure determination of protein and biological macromolecules. To perform X-ray crystallography, a lectin crystal is mounted between an X-ray source and an X-ray detector. The crystal lies in the path of a narrow beam of X-rays. The crystal diffracts the X-ray beam into many discrete beams, which produces distinct spots called reflections on the detector (Rhodes, 2000) (Figure 13). The resulting diffraction patterns yield information about crystal packing symmetry and the size of the repeating unit that forms the crystal. The intensities of the spots can be used to determine the "structure factors" from which a map of electron density can be calculated, once phases have been estimated.

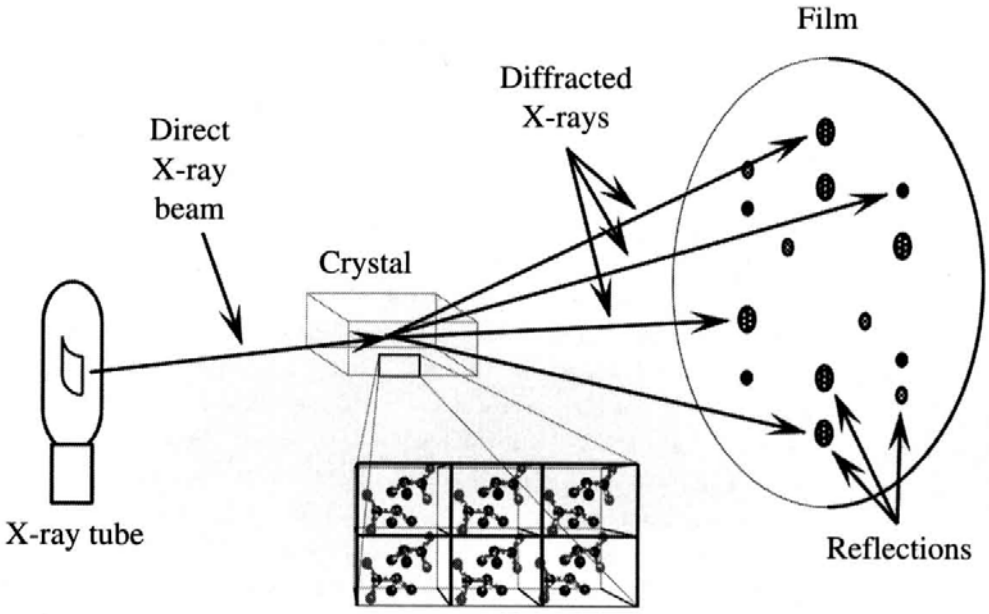

Figure 13. Crystallographic data collection.
Source: Rhodes (2000)

Various methods can be used to improve the quality of this map until it is of sufficient clarity to permit the building of the molecular structure using protein sequence. At last, the resulting structure is then refined to fit the map accurately and favored conformation was elucidated (Smyth and Martin, 2000).

For this study, trial exposures of the new lectin crystals were performed as X-ray diffraction experiments, which were carried out at 100 K using the Synchrotron Radiation Source at the Daresbury Laboratory, U.K., and charge-coupled device (CCD) on station 14.1 (Appendix H). The crystals were mounted in the beam and adjusted carefully on a device known as a goniometer in a stream of liquid nitrogen at 100K. After the exposure, X-ray diffraction images were collected on the CCD. The exposure read out a digitized image to the controlling workstation.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Mushroom collection and identification

Mushroom surveys were conducted in four provinces; Nakhon Ratchasima, Burirum, Nakhon Pathom, and Kanchanaburi, during September to October, 2002 and June to August, 2003. (Appendix D) The collection was mainly emphasized in Nakhon Ratchasima Province.

Mushroom specimens were investigated from natural forests in the suburb area, local markets around Muang District, Pak Thong Chai and Wang Num Keaw District, and Nong-rawieng Plant Genetics Forest in Nakhon Ratchasima Province. High mushroom diversity and quantities were also obtained from several mushroom markets on the roadside at Nong Ki, Nang Rong, Chalermprakiat, and Lam Plai Mat District, Burirum Province. Mushrooms collected from Nakhon Pathom were typically edible mushroom from Arunyik Farm which was located at Sam Pran District. Only a few specimens were collected at Kanchanaburi.

A total of two hundred and eighteen mushroom specimens (175, 30, 11, and 2 specimens from Nakhon Ratchasima, Burirum, Nakhon Pathom, and Kanchanaburi Provinces respectively) were collected and identified (Appendix D). Totally 48 genera of 23 families were recorded. The proportion of mushroom specimens collected from 23 families were *Agaricaceae*, *Amanitaceae*, *Auriculariaceae*, *Bolbitiaceae*, *Boletaceae*, *Cantharellaceae*, *Cariolaceae*, *Clavariaceae*, *Coprinaceae*,

Entolomataceae, Geastraceae, Helvellaceae, Hymanochaetaceae, Lycoperdaceae, Peniophoraceae, Pleurotaceae, Podoscyphaceae, Polyporaceae, Pluteaceae, Russulaceae, Schizophyllaceae, Sclerodermataceae, and *Tricholomataceae* (Figure14).

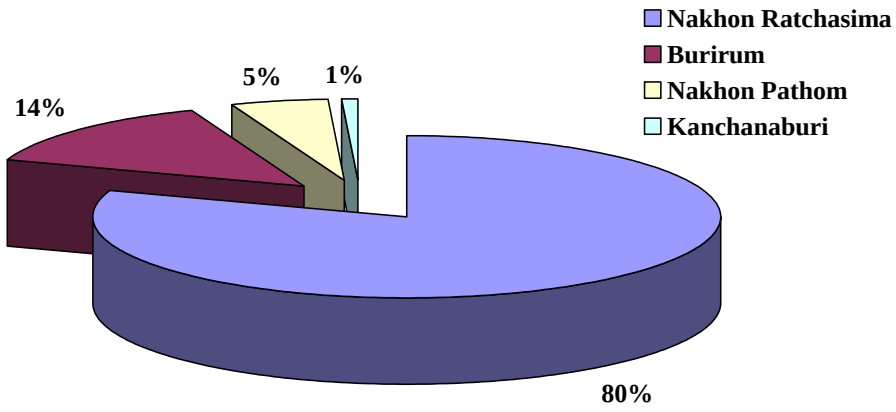

Figure 14. Total percentages of collected mushroom specimens from different collection areas in 4 provinces of Thailand.

In this study, the most frequently encountered basidiomycorta were *Tricholomataceae* (44) followed by *Russulaceae* (38) *Agaricaceae* (32), *Plurotaceae* (27), *Amanitaceae* (17), and *Boletaceae* (13). The widespread genera of mushrooms in each area of collections are shown in Table 4.

Table 4. Biodiversity of macrofungi investigated in the study in four provinces of Thailand collected during September-October 2002 and June-August 2003.

Location of Mushroom Collection	Agaricaceae	Amanitaceae	Auriculariaceae	Bolbitiaceae	Boletaceae	Cantharellaceae	Cariolaceae	Clavariaceae	Coprinaceae	Entolomataceae	Geastraceae	Helvellaceae	Hymanochaetaceae	Lycoperdaceae	Peniophoraceae	Pleurotaceae	Podoscyphaceae	Polyporaceae	Pluteaceae	Russulaceae	Schizophyllceae	Sclerodermataceae	Tricholomataceae
Nakhon Ratchasima	30	13	2	2	12	10	2	2	2	1	1	1	1	4	4	20	-	4	1	26	-	3	33
Burirum	2	4	1	-	1	2	-	-	-	-	-	-	-	2	-	-	1	-	-	12	-	-	5
Nakhon Pathom	-	-	2	-	-	-	-	-	-	-	-	-	-	-	-	7		-	-	-	2	-	-
Kanchanaburi	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	2

From the information from Table 4, they showed clearly that Nakhon Ratchasima provided a highest quantity and diversity of mushroom collection in this study. This can be described by a number of mushroom collections in this province that was high and much more frequent than other far away provinces (Burirum, Nakhon Pathom, and Kanchanaburi). High diversity of mushrooms collected in Nakhon Ratchasima intensively obtained from the Nong-raweing Plant Genetics forest. Most genera found were similar and correlated to the previous investigation (Rodtong *et al*., 1997). No substantially change was observed within the last seven years. So it demonstrated that because of high quantity of fungal specimens were provided, the study of their metabolites could possibly be investigated in this work.

Some of the mushroom species found in this study, in particularly edible mushrooms are also commonly found in both North America and Europe. This may suggest us a clue about geological history of the area and also population migration across the world (Watling, 1998). The largest number of mushroom collection at Nakhon Ratchasima were obtained from genera *Russula* and *Termitomyces* which are edible mushrooms as similar to mushrooms found from Burirum Province. The prevalent species found through Nong-raweing Plant Genetics forest was yellowish edible mushrooms, *Cantharellus minor* Peck.

The diversity of the mushroom family, *Boletaceae* in Nakhon Ratchasima Province was impressive since 5 genera; *Boletus*, *Gyroporus, Strobilomyces*, *Tylopilus*, and *Xerocomus*, were found. These genera could be classified mainly by their fruit body shape and pore color. The genus *Russula*, a mycorrhizae mushroom found in this study, was quite complex as they were reported to be found in several parts of the world (Walting, 1998). Even through the entire morphological look was

similar with the fruit body shape and also cap colour, the microscopic examination showed that they were unrelated taxa. So this confirmed clearly that the microscopic task was an important and a necessary tool for a precision of mushroom identification and classification (Bon, 1987).

Some species of mushrooms in the family; *Agaricaceae* which including genera *Leucocoprinus, Leucoagaricus, Lepiota,* and *Macrolepiota,* were also our interest since they have been reported that are high in lectin accumulation. Interestingly, these genera were found highly widespread at suburb areas of Nakhon Ratchasima. The mushroom family, *Peniophoraceae,* was greatest afforded from Pak Thong Chai District, Nakhon Ratchasima. From the reason that they are quite drier and more rigid than other mushrooms so they can be kept for a long time and only short time of air-drying was needed in the further purpose of natural product extraction. However, they were difficult to blend resulting in the poor purification.

It was also noticed from Table 14 that there were three mushroom genera *Hiemiella, Mycoamaranthus,* and *Podoscypha,* which were not found in the area of Nakhon Ratchasima and restricted only to Burirum. Some different interesting species were also collected from this province.

Since Thailand has been demonstrated as the potential country of great mushroom diversity because it locates in the junction of several natural highways of linking south and north and thereon westwards, *Heimannomyces* which is the newly blue–spored mushroom previously reported in Malaysia (Walting, 1998). This species was also presently observed in this study at the Nong-raweing Plant Genetics Forest, Nakhon Ratchasima.

Since only a few specimens were collected from Kanchanaburi in the year 2002

and no more mushroom surveys were undertaken in 2003. Heavy flooding in the year 2002 destroyed a lot of trees and also mushrooms in the forests at Kanchanaburi.

4.2 Determination of lectins accumulated in mushroom specimens

For the detection of lectins, crude extracts of 218 mushroom specimens were tested for hemagglutination activity. One hundred and twenty eight extracts were found to exhibit their specific agglutinating activity against different types of red blood cells (RBCs) (Figure 15; Appendix E). The hemolytic activity and partial hemolysis of mushroom extracts against human and animal erythrocytes were also detected at approximately 39.44% (86 out of 218 extracts). Some partial hemolysis were found at about 18.34% (40 out of 218). About 60% of the total 128 extracts gave the positive hemagglutination tests were observed to be dominantly agglutination for rat erythrocytes. Highest positive hemagglutination were observed when tested against rat erythrocytes at around 88%. The proportion of positive hemagglutination test of crude extracts from each mushroom family collected in Nakhon Ratchasima and Burirum Provinces tested against human and animal red blood cells are shown in Figures 16 and 17.

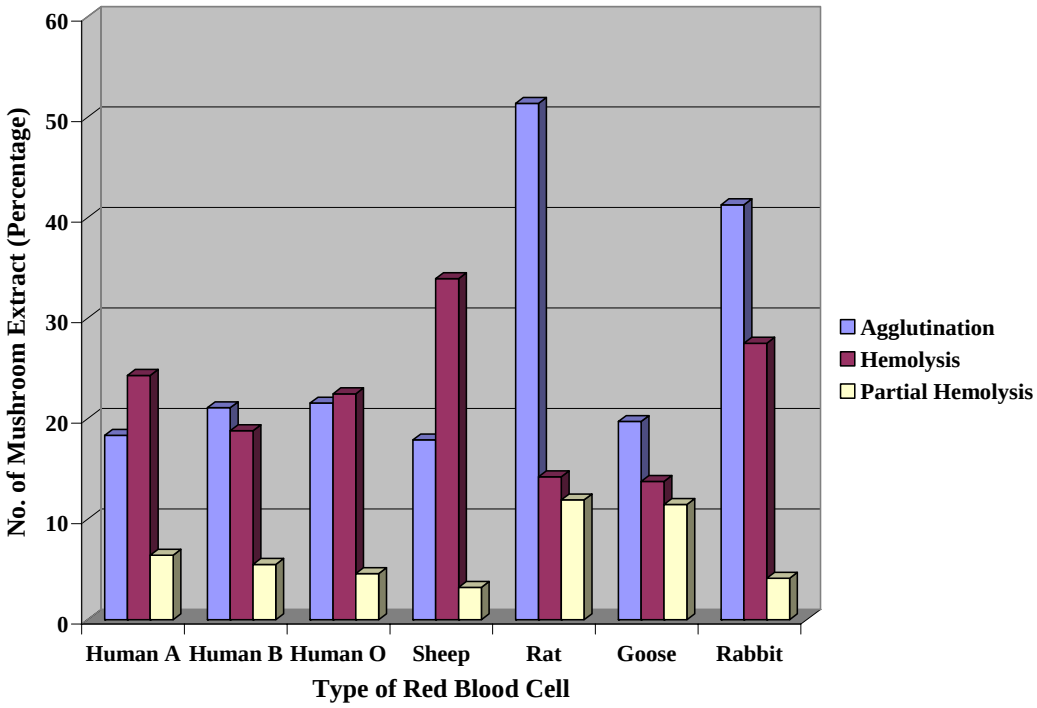

Figure 15. Proportion of positive hemagglutination test and hemolytic reaction of 86 mushroom extracts against human and animal red blood cells.

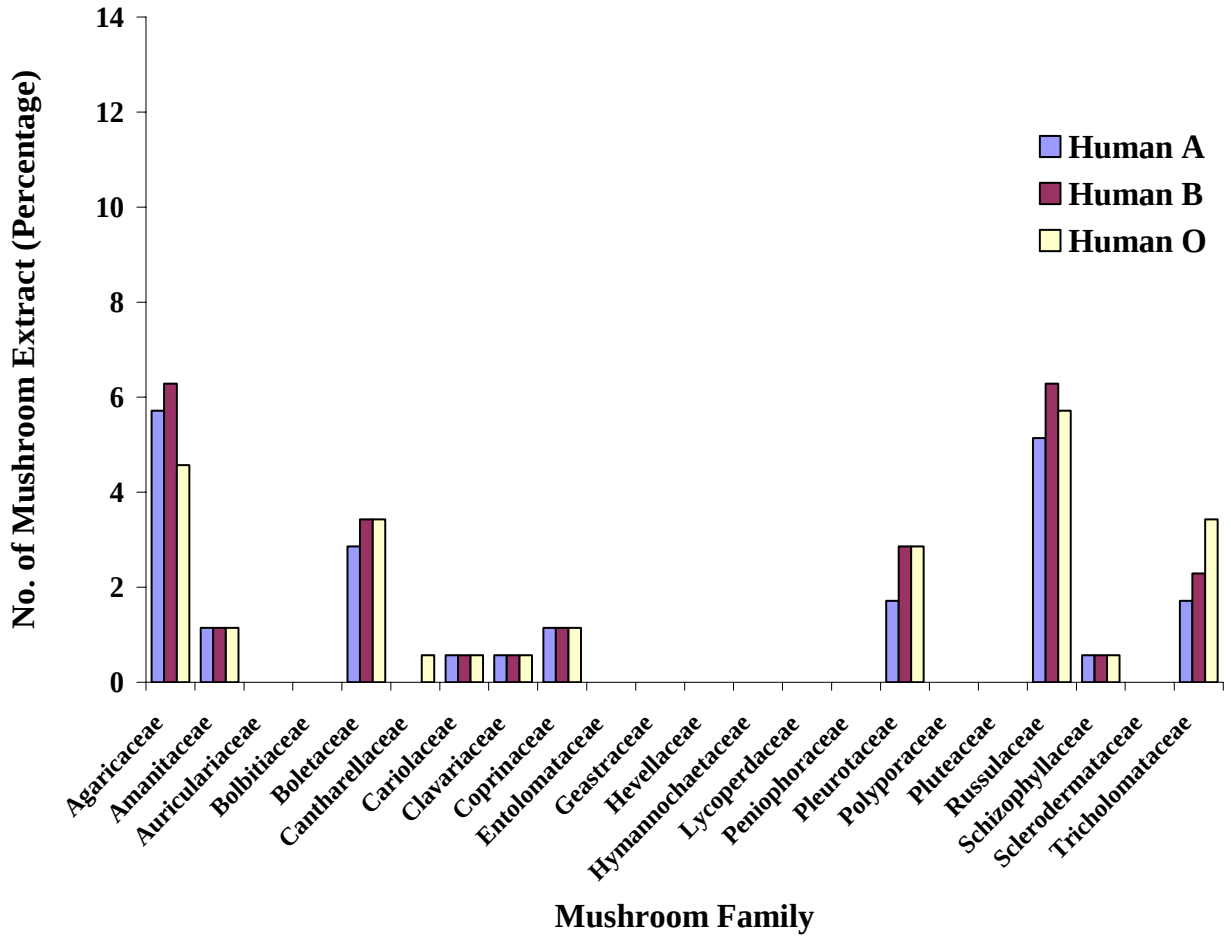

Figure 16a. Proportion of positive hemagglutination test against human red blood cells of crude extracts of mushroom collected from Nakhon Ratchasima Province.

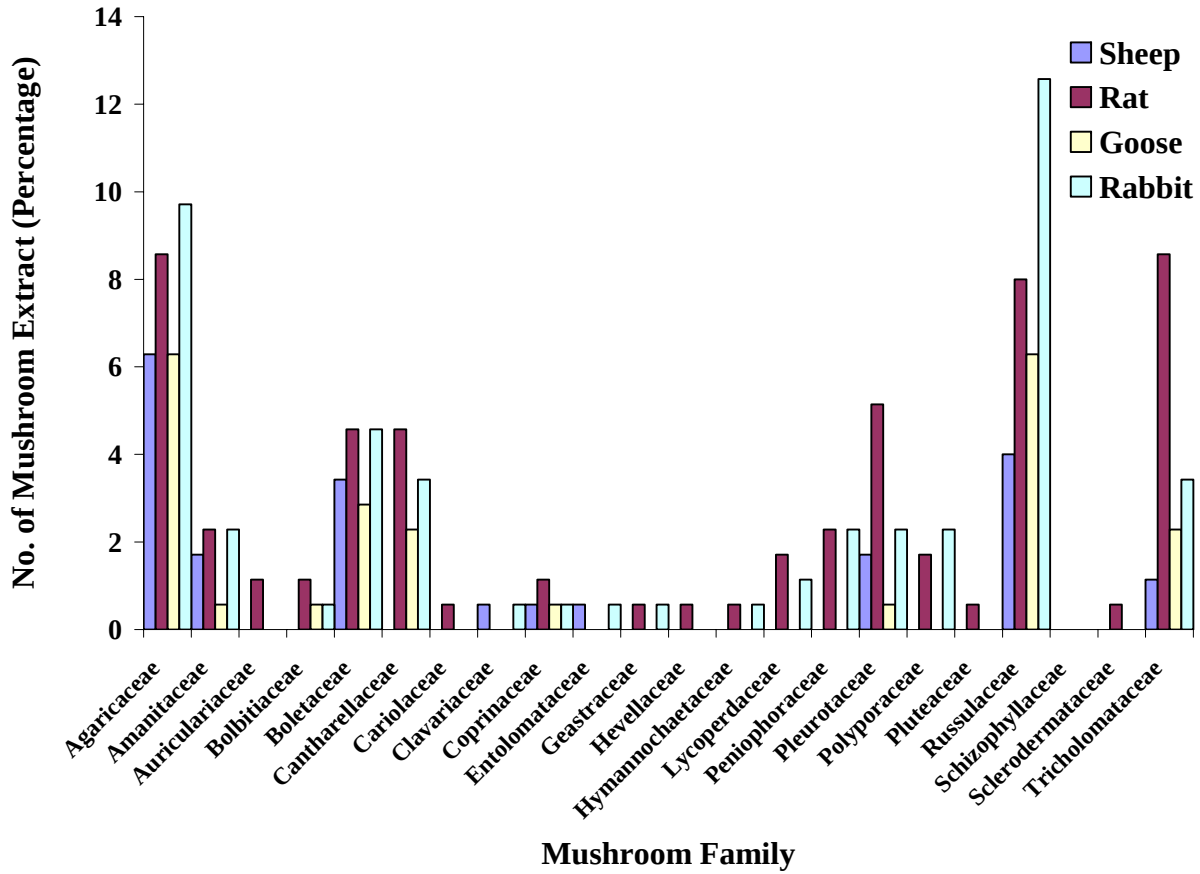

Figure 16b. Proportion of positive hemagglutination test against animal red blood cells.of crude extracts of mushroom collected from Nakhon Ratchasima Province.

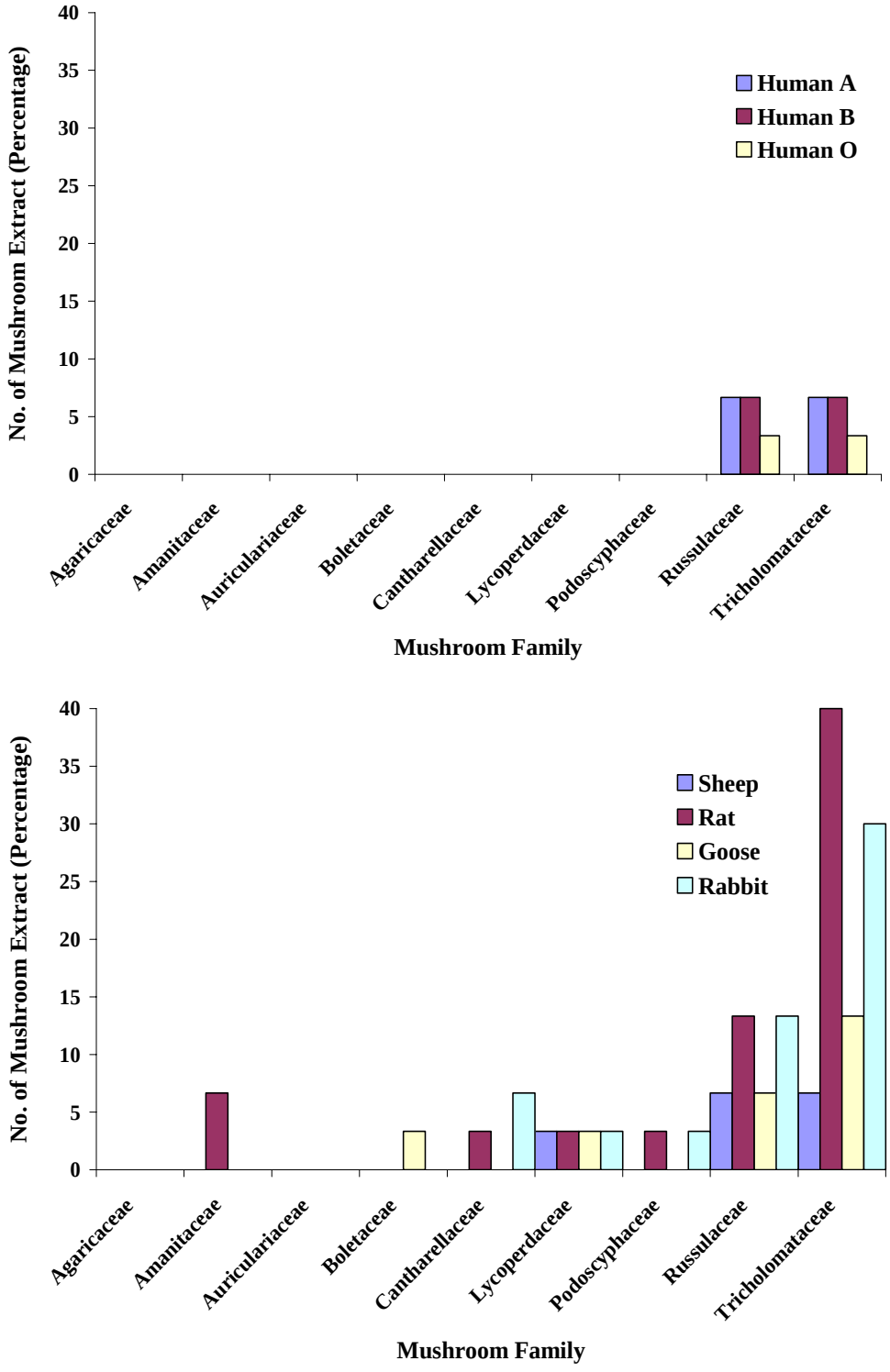

Figure 17. Proportion of positive hemagglutination test against human and animal red blood cells of crude extracts of mushroom collected from Burirum Province.

The hemagglutination tests showed that the extracts determined could be categorized into seven groups according to their specificity with RBCs. Groups one to three were active against human RBCs in ABO system (Appendix E) while group four to group seven displayed their specificity against specific types of animal RBCs. The first group was anti-A positive, which belonging to genera; *Amanita, Agaricoid, Psathyrella, Boletus, Chamaemyces, Clavulina, Coprinus, Hydnangia, Macrolepiota,* and *Russula.* The second group was anti-B extracts, which were the same genus group of anti-A but *Lentinellus* was included. The third group was anti-O, which consisted of *Amanita, Boletus, Cantharellus, Clavulina*. *Coprinus, Hydnangia Lentinellus, Macrolepiota, Psathyrella,* and *Russula*. Genera *Amanita, Boletus, Coprinus, Hydnaceae, Lepiota,* and *Russula* showed agglutination against all human ABO system. The fourth group was those genera, which displayed strong agglutination against sheep RBCs were *Boletus* and *Russula*. The fifth group was those which showed activity against rat RBCs were almost all genera found. It was noticed that most species that were active against rat and also rabbit RBCs. However, some of which agglutinated rabbit RBCs but had no activity for rat RBCs were commonly observed in *Russula*. The sixth group was those which strongly agglutinated goose RBCs were found in the genera, *Hiemiella* and *Russula.* The weak activities were shown in *Cantharellus*. Finally, the seventh group was extracts which had activity against rabbit RBCs performed as the largest group that were widespread in all genera.

Although lectins were widely determined among mushroom genera but their consistency was not high, and some specific lectins were more frequent in certain genera (Pemberton, 1994). In this study, the high variation of lectin accumulation

could be observed in each individual sample from different collection areas. This demonstrated clearly that geography was highly effected to lectin occurrence in the particular specimens. Moreover, the fruit body size and age might give a high variation of the lectin accumulation. Time and place of harvest were also another reason of their variation. This finding correlates to the previous report of Guillot and Konska (1997). When compared hemagglutination results obtained in year 2002 and 2003, the lower-titer agglutinins were observed in the mushroom species collected in year 2002.

This investigation showed that 60% of tropical mushrooms (218 specimens) accumulated lectin in their fruit bodied. This percentage is close to the previous lectin survey in Great Britain which found that 65% from 72 British mushroom species (Anstee, 1972, quoted in Pemberton,1994). But it showed higher percentage than that of the recent work of Pemberton (1994), who found that agglutinins accumulated at 50% of 403 species which was also higher than results of lectin studies in France and Germany. They demonstrated that agglutinins were contained in the fungi at 30% yield from over 600 species and 20% from 293 species, respectively (Guillot *et al.*, 1983, Seeger and Weildmann, 1972, quoted in Pemberton, 1994). This variation might be due to the different serological screening method used, and the number and variety of animal red blood cells investigations. However, this proved our hypothesis that the high diversity of mushrooms were available in Thailand and provided a high probability to find more lectin variety with a diverse specificity.

The high lectin titers were detected in mushroom extracts from mushroom genera *Amanita, Boletus, Cantharellus, Hiemiella, Lentinus, Macrolepiota, Marasmius,* and *Russula.* The crude extract of *Macrolepiota* sp. P113, which was collected from the

grape orchard in Nakhon Ratchasima in the year 2002 presented the highest lectin titer of 1,024 when tested against rat RBCs while the extract of *Amanita* cf. *nauseosa* (Waket.) Reid ML111 collected from Pak Thong Chai District, Nakhon Ratchasima Province in 2003 showed the same highest titers with rat RBCs. But it also showed a titer of 1,024 against human group O and rabbit RBCs. From two years of our study, *Amanita* gave a similar results for lectin-accumulating mushroom species which was high consistency and not sensitively changed with time and place of harvest. Furthermore, the highest titer of 1,024 were investigated from the extracts of *Russula luteotacta* Rea. and *Russula* sp. collected from Pak Thong Chai District, Nakhon Ratchasima Province, when tested against rabbit RBCs. *Lentinus* sp. ML055 collected from the suburb area of Nakhon Ratchasima Province also gave a high titer of 1024 when tested against rat RBCs, and *Hiemiella prot retispora* ML135 from local market in Chalermprakiat District, Burirum Province when tested again goose RBCs.

In addition, *Boletaceae* from Pak Thong Chai District, Nakhon Ratchasima Province, were observed to produce a titer of 768 when tested against both goose and rabbit RBCs. *Cantharellus* from different areas of Pak Thong Chai District and Nong-raweing Plant Genetics Forest, Nakhon Ratchasima presented different anti-animal RBCs-hemagglutinating activity. *Cantharellus cibarius* Fr. from suburb area of Nakhon Ratchasima showed the positive agglutination activity at a titer of 640 and 288 with rat and rabbit RBCs respectively but the extracts from Nong-raweing Plant Genetics Forest showed a titer of 288 and 544 against rat and rabbit RBCs respectively, and also showed some partial hemolysis a titer of 24 with sheep RBCs. Crude extracts of *Macrolepiota* and *Amanita* showed very rapid and strong reaction during the hemagglutination test. The extract of two species of *Lycoperdon* collected

from grape orchard and eucalyptus in the plantation area of Nakhon Ratchasima, gave the similar specific activity dominantly against rat RBCs with a titer of 256 and 544 respectively.

Even though the hemagglutination activity was highly demonstrated in the study, the information obtained from hemolytic activity and partial hemolysis are also interesting for the medical research application such as hemolysin production. The mushrooms which accumulated high hemolysin belonged to mushroom genera *Amanita, Entoloderma, Macrolepiota,* and *Lycoperdon.*

This preliminary screening of mushroom lectins shows clearly that a wide variety of tropical mushrooms in Thailand accumulates lectins in their fruit bodies. The lectins could be simply extracted and easily tested for their activity. More than 60% of mushroom extracts were found to predominantly perform hemagglutinating for rat RBCs. The high incidence of lectin accumulations was observed in mushroom specimens belonging to genera *Amanita, Boletus, Cantharellus, Hiemiella, Lentinus, Macrolepiota,* and *Russula.* Some crude extracts of *Amanita* sp. and *Macrolepiota* sp. rather strongly agglutinated both human and animal (rat, rabbit, goose, and sheep) RBCs, and were considered for further purification and preliminary characterization of their lectins. However, in order to obtain lectin from mushroom fruit bodies, the source of their specimens should be available.

Mushroom mycelia from genera *Russula* and *Amanita* were attempted to culture and preliminary test for the lectin accumulation. Unfortunately, the lectin content of those representative mushroom was much lower than from their fruit bodies and difficulty in culture isolation. Thus, only lectin accumulation in fruit bodies were focused in this study.

4.3 Selection of mushroom lectins according to their biological properties

4.3.1 Cytotoxicity test

After the preliminary hemagglutination test, 60 mushroom extracts which showed high lectin accumulation were submitted to cytotoxicity tests against both human epidermoid and human cervical carcinoma cells. From a total of 60 mushroom extacts, more than 90% exerted some cytotoxic actions on human epidermoid and cervical carcinomas. Cytotoxicity activities against both carcinoma cells were in the same direction. The highest antiproliferation activity on human epidermoid carcinoma was observed on *Amanita* sect. *Vaginatae* ML020, and followed by *Strobilomyces mollis* Corner gp. ML034, *Lentinus* sp. ML055, *Russula* sp. ML054, and *Cantharellus* cf. *cibarious* Fr. ML016 with IC_{50} of 2.5, 3, 8, 21, and 3.55 µg/ml respectively (Table 5) whereas mushroom extracts which displayed the highest antiproliferation activity on human cervical carcinoma belonged to *Lentinus* sp. ML055, *Amanita* sect. *Vaginatae* ML020, *Macrolepiota* sp. ML106, *Marasmius* sp. ML071, and *Russula* sp. ML054 with IC_{50} of 7, 0.14, 15, 9.5, and 21 µg/ml respectively. Morphological characteristics of these mushrooms which gave the high cytotoxicity activity in this test are shown in Figure 18. Compared with results from previous studies, the activity of these mushroom extract was apparently high.

Most of these mushrooms are edible. Since the well-known of anticancer mushroom genus *Lentinus* was tested, So this work demonstrated that traditional knowledge about this mushroom by local people is correct (Chamratpan, 2003), 2003). it could be confirmed by this work for their efficacy. It is also interesting that the popular edible mushroom, *Cantharellus* cf. *cibarious* Fr. which was available in

high quantity in the local area of Northeastern, Thailand, showed antiproliferation against human epidermoid carcinoma. This is good for drug development since epidermoid carcinoma currently appears to be a major cause of cancer in patients in Thailand.

Table 5. Some selected mushroom extracts for cytotoxic activity test against cancer cell lines by MTT colorimetric assay.

Extract code.	**Mushroom species**	**IC_{50} (µg/ml)**	
		KB	**HeLa**
ML016	*Cantharellus* cf. *cibarius* Fr.	3.55	3.55
ML020	*Amanita* sect. *Vaginatae*	2.5	0.14
ML034	*Strobilomyces mollis* Corner gp.	3	36.5
ML054	*Russula* sp.	21	21
ML055	*Lentinus* sp.	8	7
ML071	*Marasmius* sp.	30	9.5
ML078	*Schizophyllum commune* Fr.: Fr	20	350

KB= Human epidermoid carcinoma

HeLa= Human cervical carcinoma

Figure 18. Morphology of some tropical mushrooms which provide potential antiproliferation activity against human epidermoid and cervical carcinomas in their crude extracts; a, *Amanita* sect. *Vaginatae* ML020; b, *Strobilomyces mollis* Corner gp. ML034; c, *Lentinus* sp. ML055; d, *Marasmius* sp. ML071; e, *Russula* sp. ML054; f, *Cantharellus* cf. *cibarious* Fr. ML016.

4.3.2 Antimicrobial activity

According to the quantity of the fruit body collected from mushroom, *Clavulina cristata* ML159, *Termitomyces* sp. ML056, *Termitomyces microcarpus* ML057, *Lepista sordida* ML102, *Macrocybe* sp. P011, *Microlepiota* sp. ML114, *Microporus* sp. ML006, *Mycoamaranthus* sp. ML058, *Schizophyllum commune* ML078, *Stereum* sp. ML005, were selected to test for antimicrobial activity. Since the mushroom extraction method used for their antimicrobial test was modified from the previous method that was used in hemagglutination screening procedure, all of those crude extracts were subjected to hemagglutination assay against rat blood cells in order to confirm that they contained lectin before testing. The hemagglutination results were recorded in Table 6 and Figure 19.

Table 6. Ten different selected mushroom extracts for antimicrobial activity tests.

Extract code	Mushroom species	Titer
P011	*Macrocybe* sp.	1024
ML005	*Stereum* sp.	128
ML006	*Microporus* sp.	512
ML056	*Termitomyces* sp.	16
ML057	*Termitomyces microcarpus* (Berk. et Br.) Heim	256
ML058	*Mycoamaranthus* sp.	144
ML078	*Schizophyllum commune* Fr.: Fr.	16
ML102	*Lepista sordida* (Fr.) Sing.	32
ML114	*Macrolepiota* sp.	768
ML159	*Clavulina cristata* (Fr.) Schroet.	768

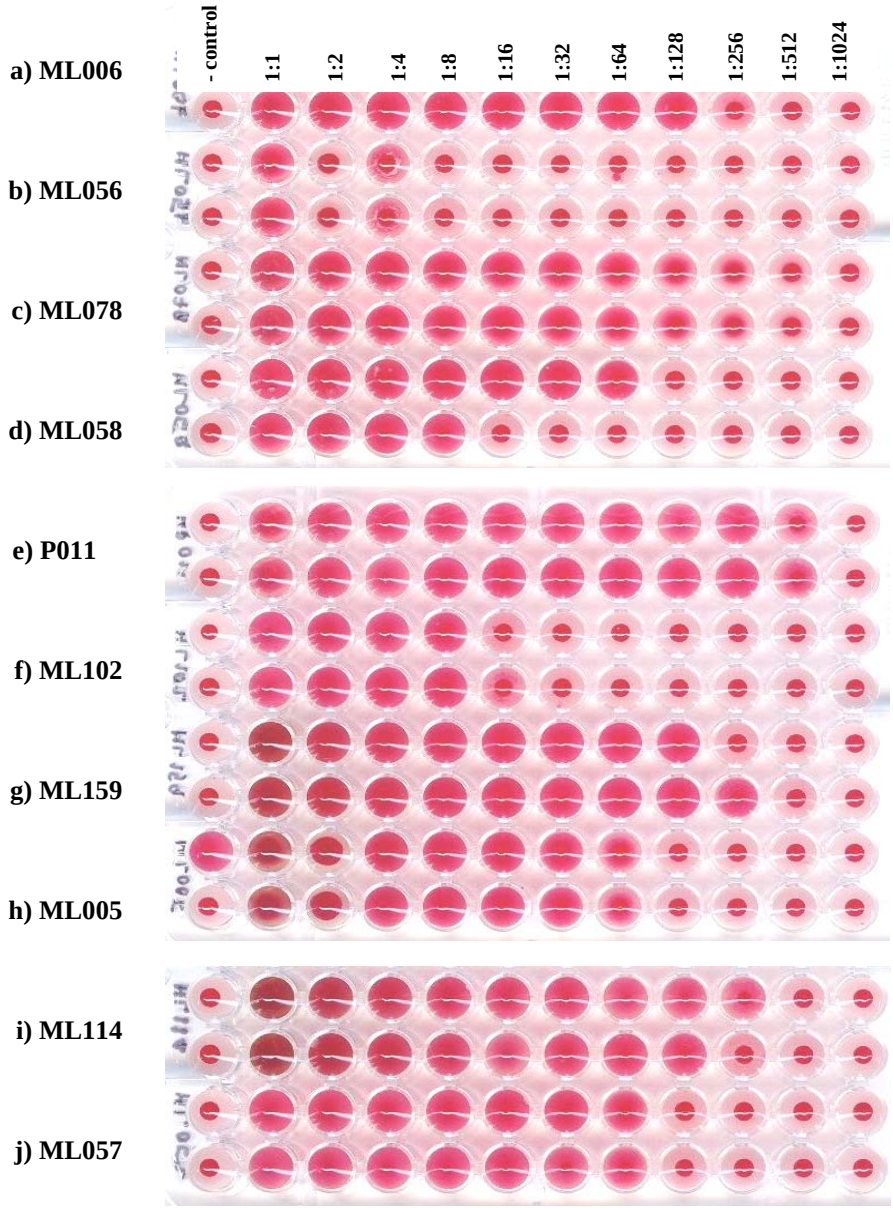

Figure 19. Hemagglutination assay of ten mushroom samples in duplicates, which were tested for their antimicrobial activity; a, ML006; b, ML056; c, ML078; d, ML058; e, P011; f, ML102; g, ML159; h, ML005; i, ML114; j, ML 057 respectively.

From the agar disc diffusion assay results, only four mushroom extracts, ML006, ML057, ML078 and ML005, showed antimicrobial activity. The crude extracts of mushroom ML006 showed considerable activity against the Gram-negative bacterium, *Bacillus cereus* ATCC 6633 and the fungus *Aspergillus niger* ATCC 6275. The extract ML057 had inhibitory activity with the Gram-positive bacterium, *Staphylococcus aureus* ATCC 29213. ML005 showed positive results against both *Staphylococcus aureus* ATCC 29213 and the yeast *Saccharomyces cerevisiae* ATCC 26108. Furthermore, extracts ML078 inhibited the growth of *Aspergillus niger* ATCC 6275. When compared to ampicillin activity (10 mg/ml), mushroom extracts no. ML006 gave larger inhibition zone than ampicillin, whereas other extracts had narrow zones (Table 7).

The inhibition zone of positive control was 100% which calculated from Table 8. The crude extract from *Termitomyces microcarpus* ML057 against *Staphylococcus aureus* ATCC 29213, was 29.2% comparing with positive control (Table 8, Figure 20). The inhibition zone of the crude extract from *Stereum* sp. ML005 against *Staphylococcus aureus* ATCC 29213 and yeast *Saccharomyces cerevisiae* ATCC 26108 were 33.3% and 59.4% comparing with positive control of amplicillin and nystatin respectively (Figure 21). The inhibition zone of the crude extract from *Microporus* sp. ML006 against *Bacillus cereus* ATCC 6633 and the fungus *Aspergillus niger* ATCC 6275 were 115% and 60.45% comparing with positive control of amplicillin and nystatin (100 Units) respectively (Figure 21). For *Schizophyllum commune* ML078, the inhibition zone of the crude extract against *Aspergillus niger* ATCC 6275 was only 36.36% (Figure 20).

It was recorded that some lectins may manifest antifungal activity, e.g. lectins from red kidney bean and potato. Other lectins from sugar snap legumes were devoid of antifungal activity (Ng and Lam, 2002). Since mushroom lectins have become of great interest, some antifungal lectin studies were carried out. However, *Agrocybe cylindracea* lectin, *Agaricus bisporus* lectin, and *Lyophyllum shimeiji* agglutinin were devoid of the activity (Ng and Lam, 2002).

Table 7. Antibacterial activity of the selected mushroom extracts against bacteria and fungi using the agar disc diffusion assay.

Mushroom extract code	**Inhibition zone diameter (mm)**						
	1	**2**	**3**	**4**	**5**	**6**	**7**
ML005	14.5	-	-	9.5	-	-	-
ML006	-	11.5	-	-	-	13.3	-
ML011	-	-	-	-	-	-	-
ML056	-	-	-	-	-	-	-
ML057	12.7	-	-	-	-	-	-
ML058	-	-	-	-	-	-	-
ML078	-	-	-	-	-	8	-
ML102	-	-	-	-	-	-	-
ML114	-	-	-	-	-	-	-
ML159	-	-	-	-	-	-	-

1= *Staphylococcus aureus* ATCC 29213, 2= *Bacillus cereus* ATCC 6633,
3= *Escherichia. coli* ATCC 25922, 4= *Saccharomyces cerevisiae* ATCC 26108,
5= *Candida albican* ATCC 10231, 6= *Aspergillus niger* ATCC 6275,
7= *Pencillium digitatum* ATCC 201167

Table 8. Antibacterial activity of ampicillin (10 mg/ml) and nystatin (100 Units) against bacteria and fungi.

Antibiotics	**Inhibition zone diameter (mm)**						
	1	**2**	**3**	**4**	**5**	**6**	**7**
Ampicillin	43.5	10	28.5	-	-	-	-
Nystatin	-	-	-	16	20	22	22

1= *Staphylococcus aureus* ATCC 29213, 2= *Bacillus cereus* ATCC 6633,
3= *Escherichia. coli* ATCC 25922, 4= *Saccharomyces cerevisiae* ATCC 26108,
5= *Candida albican* ATCC 10231, 6= *Aspergillus niger* ATCC 6275,
7= *Pencillium digitatum* ATCC 201167

Fungal lectin which reported to have the antibacterial activity was also relatively few. From this study, *Microporus* sp. ML006 extract showed it's great activity against *Aspergillus niger* ATCC 6275 comparing to other extracts. This should be useful for future application. Thus this work helps to shed light on the discovery of antimicrobial lectins. However, to utilize these lectins, the intensive study of antimicrobial mushroom lectins should be performed. Perhaps if these biological activities were emphasized, the physiological functions of mushroom lectins could be elucidated. The morphology of mushroom containing these biologically active extracts is shown in Figures 22, 23, 24, and 25.

Although only low antimicrobial activity was obtained from these mushroom lectins, this information is still promising and important for future research because, nowadays, the direct extraction of bioactive compounds from their natural source is not the only way for the lectin investigation. If the structure of the bioactive compound was elucidated, a synthetic compound could be possibly produced by using knowledge on recombinant DNA technology. Since antimicrobial-resistant organisms have been a major problem in medical treatment, searching for novel antimicrobial compounds is definitely useful and important.

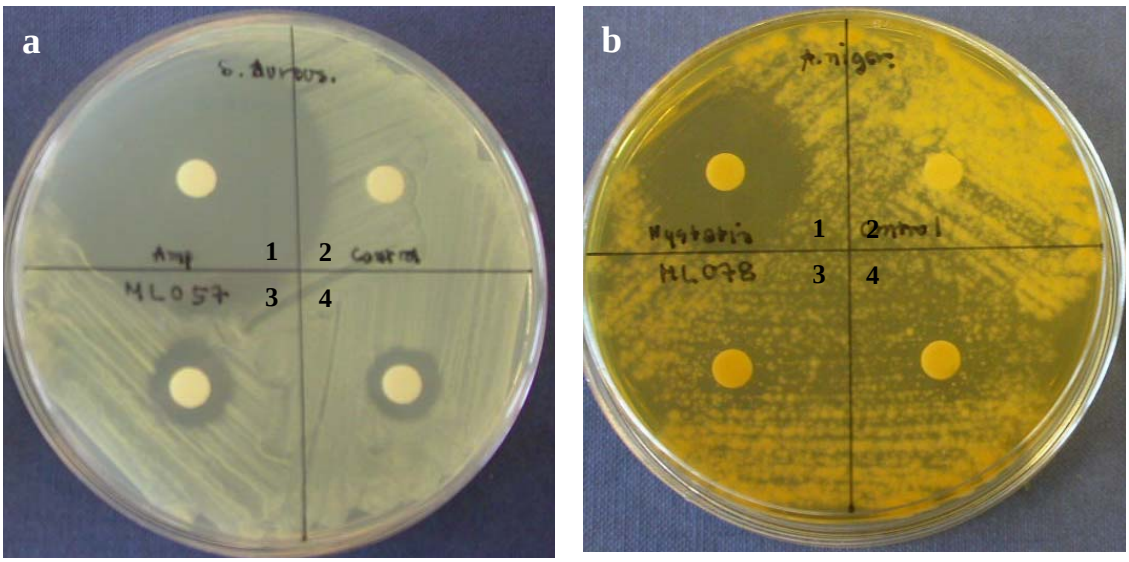

Figure 20. Antimicrobial activity of *Termitomyces microcarpus* ML057 crude extract against *Staphylococcus aureus* ATCC 29213 (a) and *Schizophyllum commune* ML078 crude extract against *Aspergillus niger* ATCC 6275 (b) after 24 and 48 h incubation respectively. The inhibition of amplicillin (1a) and nystatin (1b) show as positive control respectively.

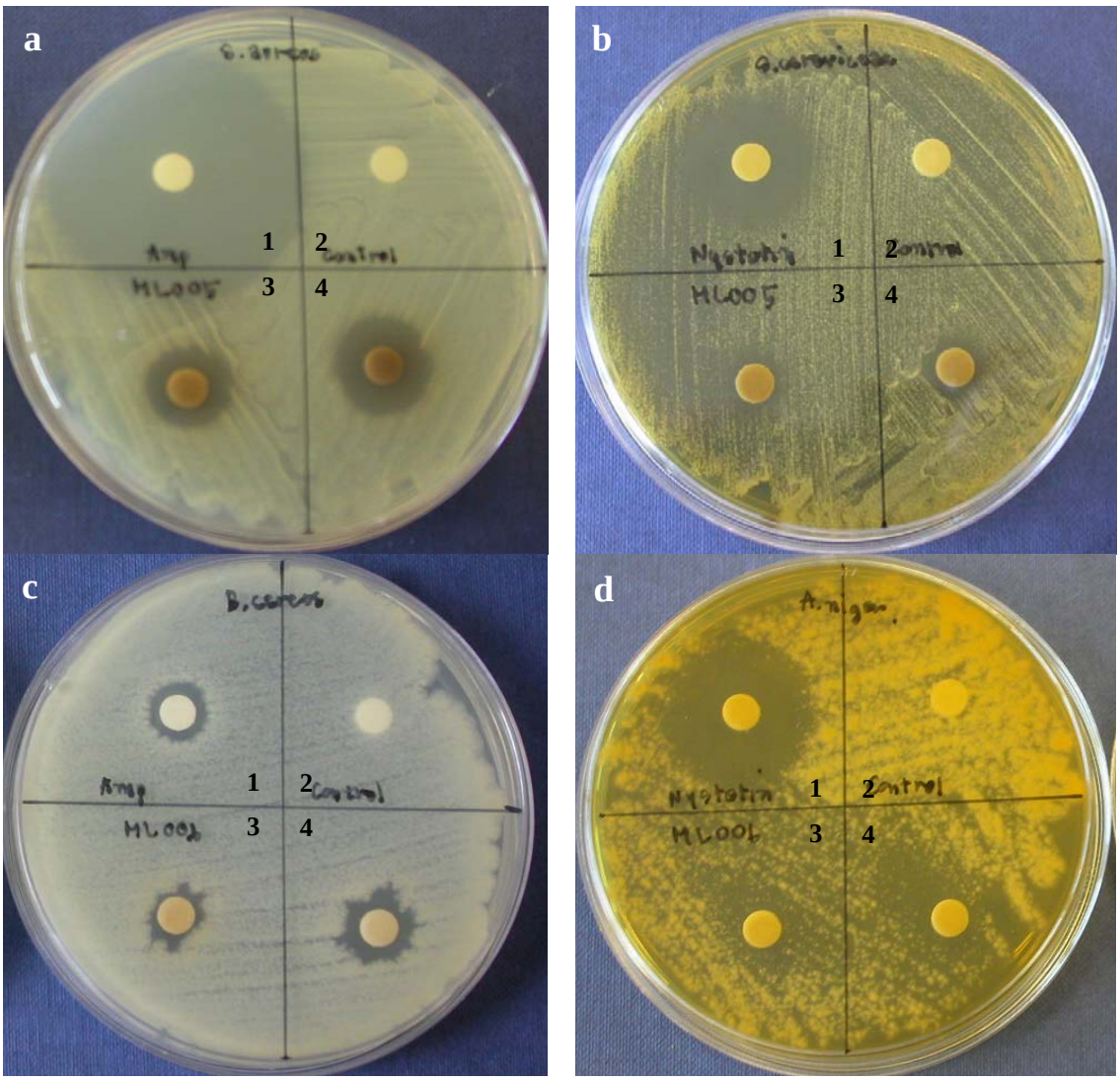

Figure 21. Antimicrobial activity of *Stereum* sp. ML005 crude extract against *S. aureus* after 24 h incubation (a) and *Saccharomyces cerevisiae* ATCC 26108 after 48 h incubation (b). *Microporus* sp. ML006 crude extract against *Bacillus cereus* ATCC 6633 after 24 h incubation (c) and *Aspergillus niger* ATCC 6275 after 48 h incubation (d).The inhibition of amplicillin (1a, 1c) and nystatin (1b, 1d) show as positive control respectively.

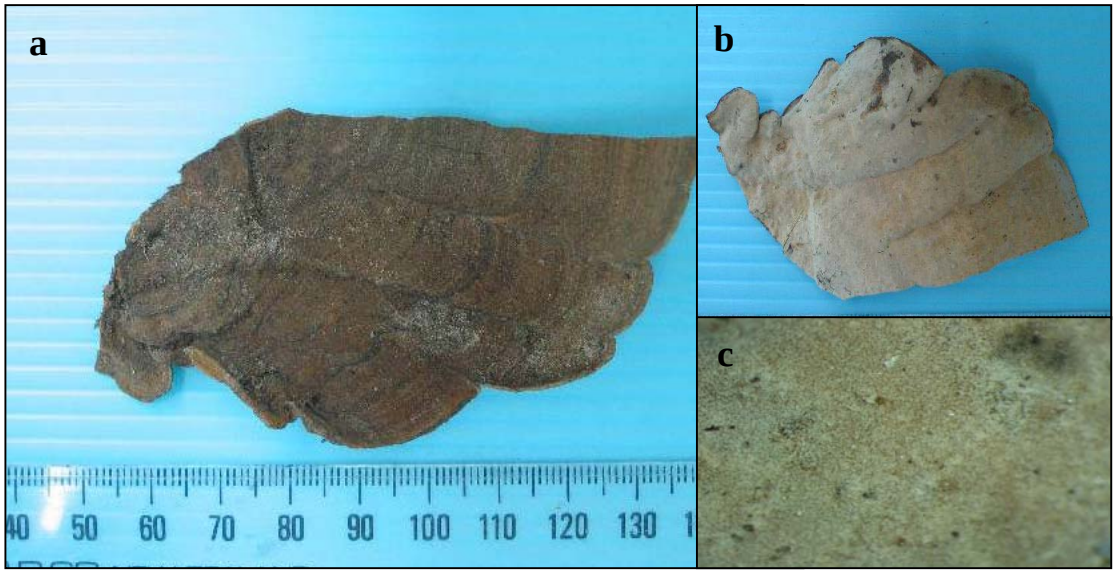

Figure 22. Fruit body of *Stereum* sp. ML005 from natural habitat (a), underneath of the fruit body (b), and underneath of the fruit body under stereomicroscope at 32 times (c).

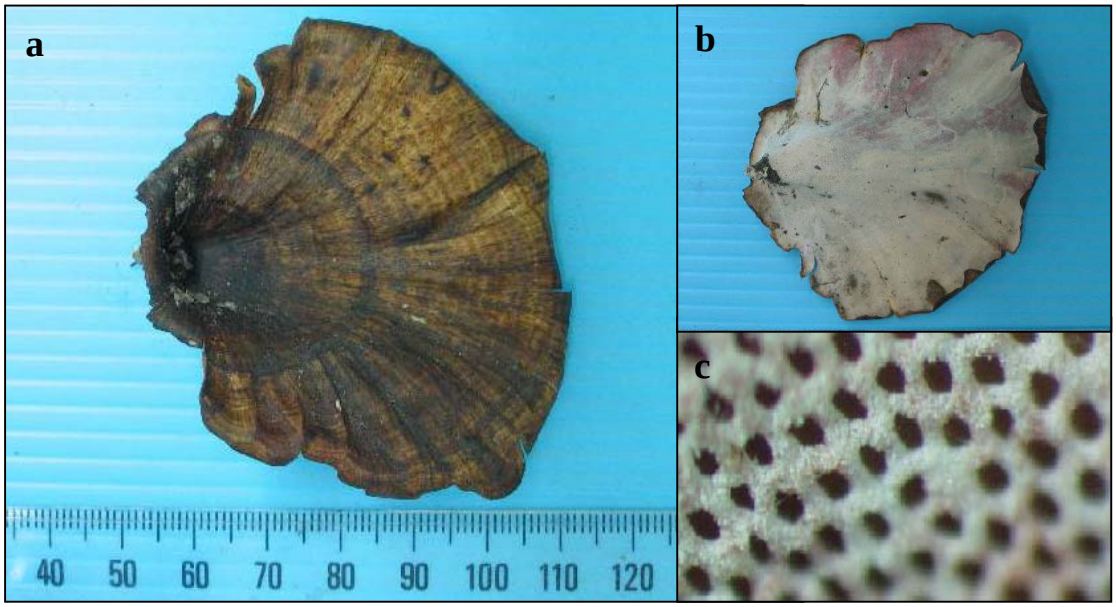

Figure 23. Fruit body of *Microporus* sp. ML006 (a), underneath of the fruit body (b), and under stereomicroscope at 32 times (c).

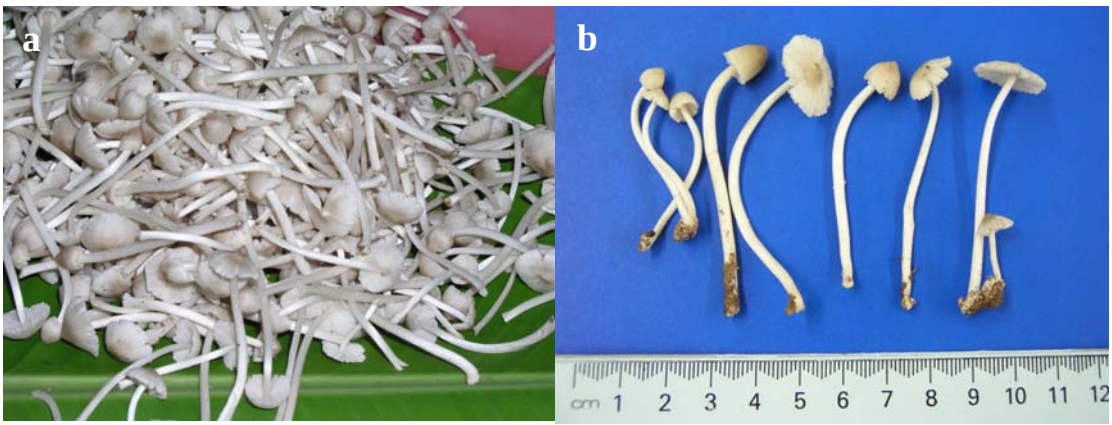

Figure 24. Fruit bodies of *Termitomyces microcarpus* ML057 (a and b).

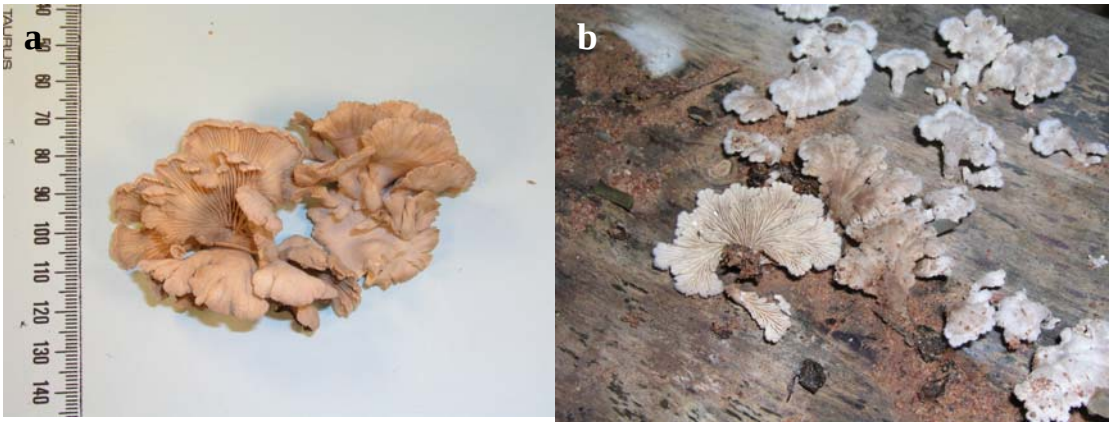

Figure 25. Fruit bodies of *Schizophyllum commune*; a) sold at the local market and b) grown in nature**.**

4.3.3 Preliminary purification of mushroom lectins

According to the results of biological activity test; the mushroom extract with antimicrobial and cytotoxicity activities were found. Seven mushroom species, *Amanita* sp. P042, *Tricholoma* sp. P011, *Termitomyces* sp. P124, *Russula* sp. P118, *Boletus* sp. P049, *Schizophyllum commune* ML078, and *Russula* sect. *plarantae* ML133, which their crude extracts showed high potency of the biological activity, were selected for lectin purification. However, to select the mushroom lectin for further purification and structure characterization, the information on purification procedure of each mushroom lectin was crucial for final decision. Thus the seven mushroom specimens; were selected as representative samples for lectin purification. In these experiments, different types of affinity column, ion exchange chromatography and also gel filtration were investigated. From all mushroom purifications, it was found that the affinity chromatography performed as the most powerful tool for these mushroom lectin purification. Only one step of the affinity chromatography column could give high purities of several mushroom lectins. In contrast, ion exchange columns (DEAE-Sapharose and CM-Sepharose) required more purification steps in combination to achieve the same purity as the affinity chromatography and gel filtration. With the gel filtration method on Sephadex G75, DNA contamination was found to be the major problem for the successful purification (data not shown).

Three affinity chromatography columns: mucin-Sepharose 4B, desialylated mucin Sepharose 4B, and N-acetyl-D-galactosamine (GalNac), were appropriated for the mushroom lectin purification from the following mushroom genera; *Amanita, Tricholoma, Termitomyces,* and *Schizophyllum,* 2) *Boletus* 3) *Russula* respectively.

In the combination of antimicrobial and cytotoxicity results, only two mushroom samples were selected for further lectin investigation. Although mushroom extracts of *Termitomyces microcarpus* ML057 gave a good result by using the affinity column, the amounts of this mushroom fruit body were not sufficient for lectin extraction and purification. Only *Schizophyllum commune* had enough quantity of fruit bodies, high accumulated lectin, and exhibited moderate antimicrobial and anticancer activity. So it was selected for the purification and characterization steps. *Schizophyllum commune* is also called the split-gill fungus. This fan-shaped fungus belongs to the genus distinguished by gill-like structures that are spit lengthways, the two parts curling inwards in dry conditions to protect the spore-bearing hymenium. Stemless, or with a short, stem like base, it is covered with grey-white felt. An electron micrograph of this spilt-gill is illustrated in Figure 26. Fruit body size is about 1-5 cm with white spores. It is able to grow on sun-baked or wind-dried wood (Figure 25b). This mushroom is edible, and has already been in commercial production (Figure 25a) (Læssøe, 1998).

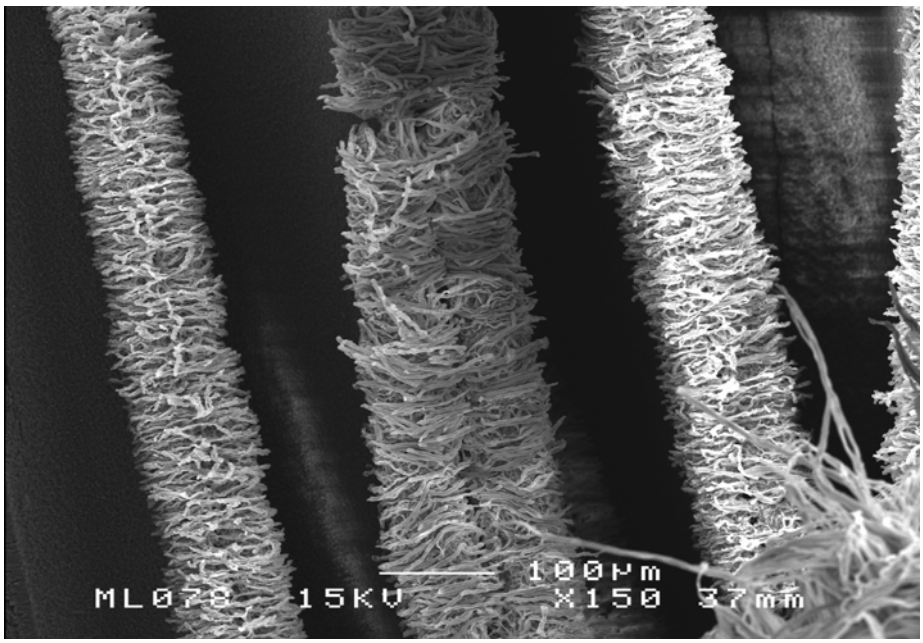

Figure 26. Scanning electron micrograph showing the split-gill of the *Schizophyllum commune*.

4.4 Purification and characterization of the selected lectin

4.4.1 Extraction and purification of mushroom lectin

Because mushrooms have high levels of phenolic compounds as well as plants, in order to prepare the extracts, 1.5% w/v of insoluble poly(vinylpyrrolidone) powder was added to the lectin extraction buffer in the first stage of purification. This is helpfully overcome the problem of polyphenols which had been previously observed to interfere with protein absorbance at 280 nm. The *Schizophyllum commune* lectin (SCL) was isolated from dried mushroom powders of fruit bodies. Initially the powder was homogenized in the extraction buffer (Appendix A) and left overnight. Then the extract was filtrated through a double layer of cheese cloth to remove any large impurities and centrifuged followed by ammonium salt precipitation at 30% saturation. In this study, salt precipitation was presented at low concentration. Ultracentrifugation was performed at the high speed on the next day. The supernatant was finally obtained, and defined as the crude extract. For lectin purification, dialysis method should be avoided to prevent lectin loss by interaction with the dialysis membrane, which is a polymer of cellulose. So the crude extract was then directly subjected to the mucin-Sepharose 4B column. The elution profiles are shown in Figures 27 and 28 respectively.

The column was washed with 10 mM Tris-HCl buffer, pH 8 but the hemagglutinating activity (bound lectin) was eluted with an unbuffer solution of 20 mM DAP. Because the DAP provided a harsh environmental condition for the lectin, the eluates were collected with 6 ml fractions and immediately neutralized with 1 ml of 1M Tris-HCl buffer, pH 7. Even after only one step of the affinity column, lectin fractions were obtained. To remove all impurities in these bound fractions, those

fractions were pooled together and rerun with the same column to ensure the purity of the lectin that would be high enough for the purpose of crystallization. More than 95% purity had to be achieved (Bergfors, 1999). Purified lectin fractions are presented as peak A in the second round of affinity chromatography Figure 28.

A summary of the purification procedure is described in Table 9. The specific activity of the crude extract was obtained as 86 units/mg, but after purification by double steps of the affinity chromatography, the specific activity of the lectin was increased up to 2,496 units/mg. From 15 g of dried mushroom powder, 32.4 mg of lectin was obtained. The lectin yield obtained from this study is quite high when compared with previous lectin purification of *Pleurotus ostreatus* (Brechtel *et al.*, 2001) and *Volvariella volvacea* (Lin and Chou, 1984).

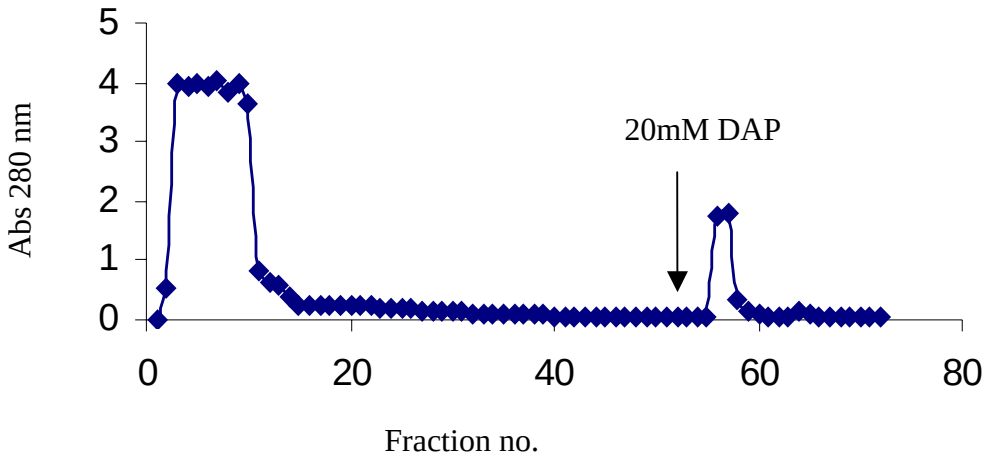

Figure 27. Purified lectin fractions of *Schizophyllum commune* on mucin-Sephorose 4B column.

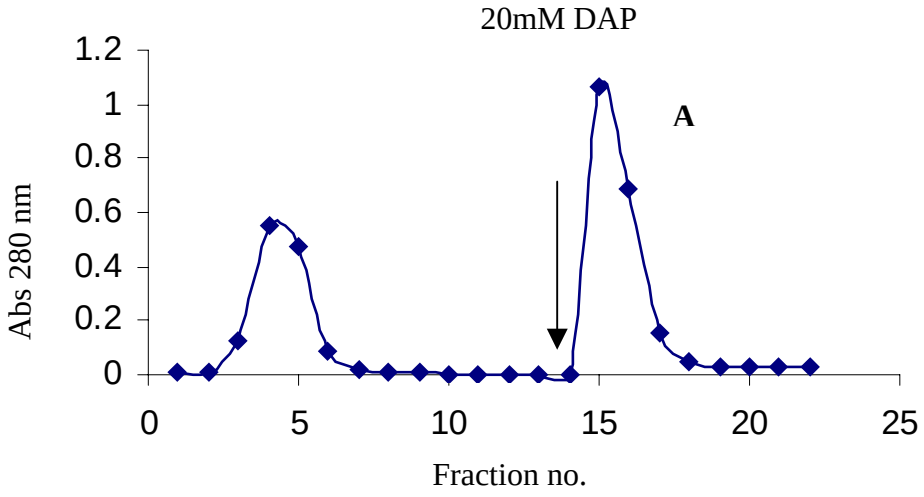

Figure 28. Repeat of the mucin-Sepharose affinity chromatography run of the bound fraction from the 1st run of crude extract through mucin-Sepharose column. Peak A was final purified lectin.

Table 9. Hemagglutinating activities of *Schizophyllum commune* lectin (SCL) chromatographic fractions from 15 g of dried mushroom powder against rabbit RBCs**.**

Step	Yield (mg)	Specific hemagglutinating activity (units/mg)	Total hemagglutinating activity (units)	Recovery of hemagglutinating activity (%)	Folds of purification
Extraction	7548	86.48	652,800	100	-
30% NH_2SO_4 precipitation	4371	87.85	384,000	58.82	1.015
Affinity column	32.30	2496.09	80,640	12.35	28.86

The use of the porcine stomach mucin-Sepharose column proved to be a suitable affinity chromatography medium for the isolation of the lectin from the tropical mushroom, *Schizophyllum commune*, and it has been successfully used for the isolation of the fungal lectin from *Rhizopus stolonifer* (Oda *et al.*, 2003). From 15 g of dried fruiting body of mushroom powder, 32.30 mg of the purified SCL was isolated. A 28-fold of purification could be obtained by this purification protocol.

Although, *Schizophyllum commune* is mushroom which is closely related to *Pleurotus ostreatus*, desialylated hog gastric mucin affinity chromatography was found to be inappropriate for its purification (data not shown). This finding suggests that a carbohydrate-specificity difference of the lectins between the two mushroom species are significant for the efficiency of the purification for crystallization purpose (Conrad and Rudiger, 1994; Chattopadhyay *et al.*, 1999).

4.4.2 Sodium dodecyl sulphate-polyacylamide gel electrophoresis (SDS-PAGE)

After TCA precipitation of lectin from all collected fractions, approximately 30 µl of protein was loaded on to the SDS-PAGE. Polyacrylamide gel electrophoresis in the presence of SDS was investigated for all purified samples from each step of the purification process. Data are shown in Figure 29.

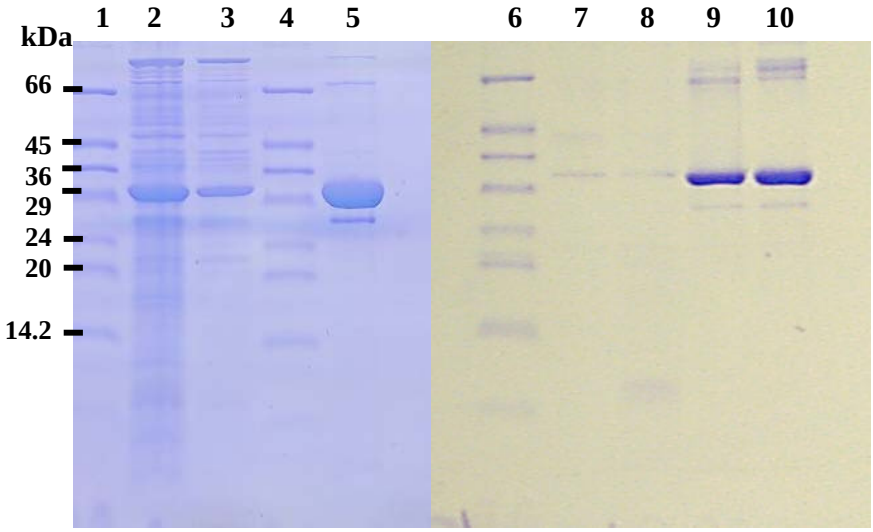

Figure 29. SDS-PAGE of *Schizophyllum commune* lectin (SCL). Lanes: 1, 4, and 6, low molecular weight standard markers (from top downward, bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), bovine carbonic anhydrase (29 kDa), bovine pancreas trypsinogen (24 kDa), soybean trypsin inhibitor (20 kDa), and bovine milk α-lactalbumin (14.2 kDa)); 2, crude extracts; and 3, after 30% ammonium sulphate precipitation; 5, 7, 8, 9, and 10 purified lectins.

The results from lanes 2, 3, 5, 9, and 10 in Figure 29 demonstrated clearly that the protein purity was consequently increasing with this sequence protein purification procedure. SDS-PAGE in the presence and absence of 2-merceptoethanol also showed the same results with a strong band of the lectin corresponding to an apparent molecular weight of 31.5 kDa and a faint band of 29 kDa were observed. Some small faint discrete bands with high molecular weight were also present, and probably be lectin dimers, similar to the results for other plant lectins e.g. bluebell bulb lectin (Wright, 1998). However, they did not interfere the subsequent crystallization process.

4.4.3 Native particle gel-PAGE

For Native-PAGE studies, the two dimensional gel electrophoresis method was modified. When the purified material was analyzed by the native gel electrophoresis (which was carried out on 29:1 acrylamide gel and run at 25 mA), the bands cut from the native gel were soaked and consequently run on SDS-PAGE for the two dimensional analysis. From the first run of the native gel, a sharp band plus a more diffuse band were obtained. After the bands of interest were cut into a gel segment and run on SDS-PAGE, the diffuse band gave two different bands consisting of a strong band which is a glycoprotein and the other weaker bands of co-purified lectin that may correspond to unglycosylated SCL. While the sharp band in another lane gave a band with higher molecular weight that may correspond to the homodimer (data shown in Figure 30).

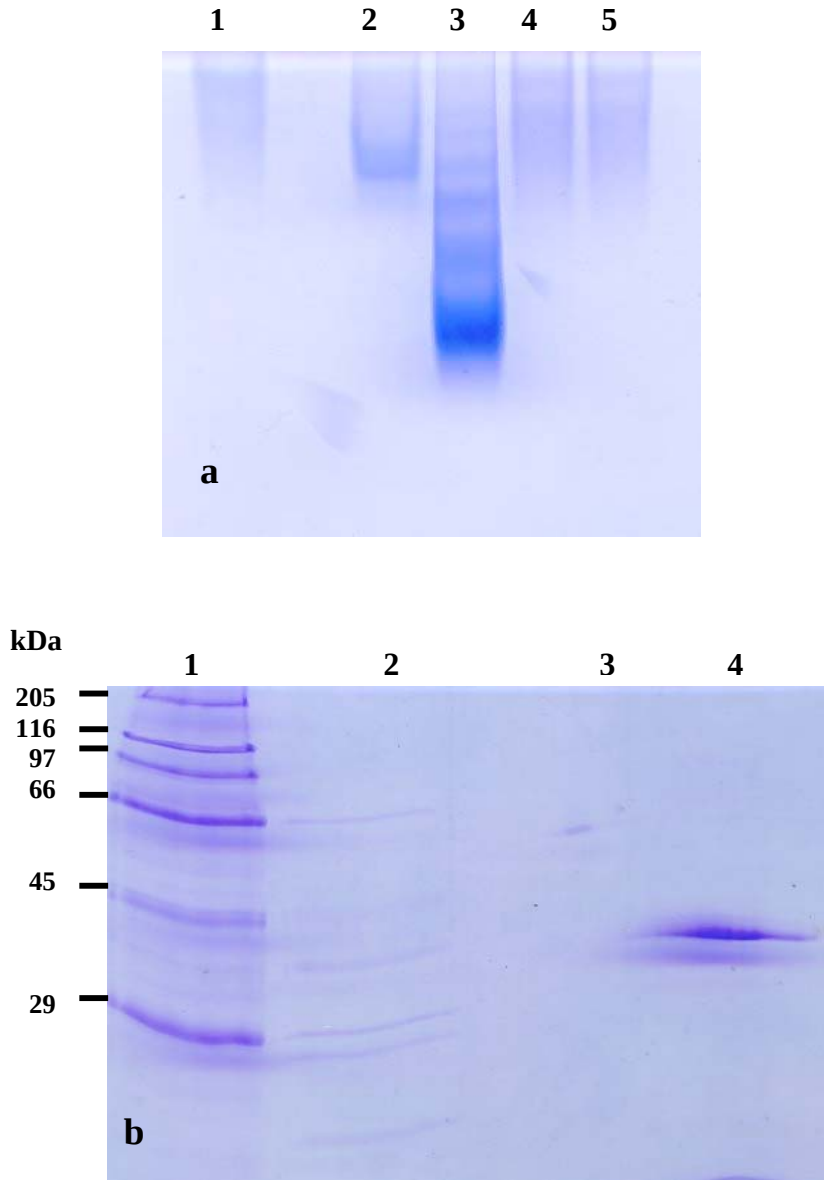

Figure 30. Molecular mass determination by two-dimension gel electrophoresis: (a) the first dimension of native particle gel-PAGE; lanes: 1, myoglobin; 2, hemoglobin; 3, bovine serum albumin; 4 and 5, purified lectin; (b) the second dimension by SDS-PAGE of purified lectin; lanes; 1, high molecular weight standard markers, HMW-SDS marker kit (Pharmacia, Sweden); 2, low molecular weight markers, LMW-SDS marker kit (Pharmacia, Sweden); 3, homodimer; and 4, purified lectin.

From results of this study, the purified SCL was characterized as a GalNAc-specific glycosylated lectin which contained a subunit with molecular weight of 31.5 kDa and forms a homodimer which could be detected in 2D gel electrophoresis. Beside that another co-purified lectins (29 kDa), which we have shown to be the unglycosylated protein, is normally seen on SDS-PAGE gel. SCL is quite similar to other mushroom lectins, in particularly *Pleurotus ostreatus* which mostly are dimers of equal or nearly equal subunits (Conrad and Rudiger, 1994). Incidentally, this molecular weight of lectin is close to fucose-recognized lectin extracted from *Aleuria aurantia*, which is composed of two identical subunits of 36 kDa (Nagata *et al.*, 1991; Wimmerova *et al.*, 2003; Fujihashi *et al.*, 2002) whereas other mushroom lectins are composed of multiple subunits, for example, *Agaricus campestris* lectin and *Hericium erinaceum* which are tetrameric (Sage &Vazquez, 1967; Kawagishi *et al.*, 1994)

4.4.4 Glycoprotein analysis using SDS-PAGE

The periodic acid schiff's (PAS) assay showed that the strong band in Figure 30 was a glycosylated protein, but probably it was not highly glycosylated. Thus only a faint pink band was noticed and compared with the strong pink colour bands of the positive controls using β2 glycoprotein I and chicken IgG which are the glycoproteins from human and chicken, respectively. This indicated that the lectin was a glycoprotein. The gel is shown in Figure 31.

Staining of SDS-PAGE gel with periodic acid Schiff's reagent yielded positive result with a faint band, indicating that the portion of carbohydrate in the molecule were much less than the portion of protein. This was a reliable method and correlated with previous reviews about carbohydrate composition of mushroom lectins

that are usually ranging from 2 up to maximum of 30% (Guillot and Konska, 1997).

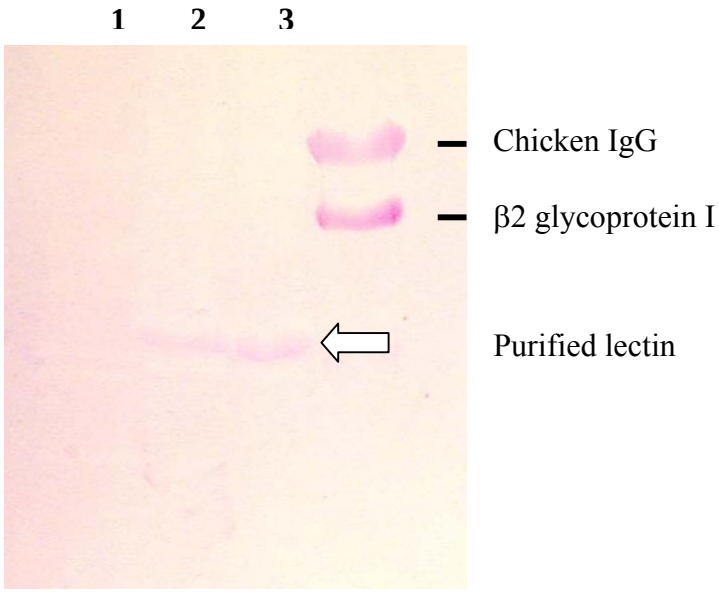

Figure 31. Schiff's periodic assay of the purified lectin (lanes 1 and 2) using β2 glycoprotein I and chicken IgG as positive controls (lane 3).

4.4.5 Sugar specificity test

According to the preliminary hemagglutination results, the crude extract of *Schizophyllum commune* was shown to agglutinate both rat and rabbit RBCs. However, it showed a little greater hemagglutinating activity with rat red blood cells. In this study the rabbit RBCs were selected for a specific activity test and sugar inhibition assay in order to facilitate comparison between the lectin activity of this SCL and other mushroom lectins that have been previously studied. In the binding-specificity assay, D-galactose, GalNAc, and lactose showed inhibitory activity against

the hemagglutination of SCL with the rabbit RBCs at 100, 0.78, and 0.78 mmol/l respectively (Figure 32). Other sugars tested, which including L-rhamnose, L-arabinose, D-xylose, D-glucose, D-mannose, and raffinose showed no inhibitory effect on the lectin even at high concentration (Table 10).

Table 10. Hemagglutination inhibition of *Schizophyllum commune* lectin (16 HA units) by various sugars.

Test sugars	Concentration of sugar in mmol/l											
	200	100	50	25	12.5	6.25	3.12	1.56	0.78	0.39	0.17	PBS
D-glucose	+	+	+	+	+	+	+	+	+	+	+	+
D-galactose	-	-	+	+	+	+	+	+	+	+	+	+
GalNAc	-	-	-	-	-	-	-	-	-	+	+	+
Mannose	+	+	+	+	+	+	+	+	+	+	+	+
Xylose	+	+	+	+	+	+	+	+	+	+	+	+
Fucose	+	+	+	+	+	+	+	+	+	+	+	+
Raffinose	+	+	+	+	+	+	+	+	+	+	+	+
Arabinose	+	+	+	+	+	+	+	+	+	+	+	+
Rhamnose	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	-	-	-	-	-	-	-	-	-	+	+	+

+ = positive result, - = negative result

This lectin could agglutinate both rabbit and rat RBCs and showed a little higher activity with rat RBCs. SCL had weak specificity towards galactose, but much stronger specificity towards GalNAc and lactose. In this respect it is similar to other previous studies of galactose-specific lectins of *Pleurotus cornucopiae* and *Pleurotus ostreatus* (Oguri *et al.*, 1996; Wang *et al.*, 2000), GalNAc-specific lectins of *Lactarious deliciosus* (Guillot *et al.*, 1991) and *Agrocybe cylindracea* (Wang *et al.*, 2002), and lactose specific mushroom lectin of *Hygrophorus hypothejus* (Veau *et al.*, 1999).

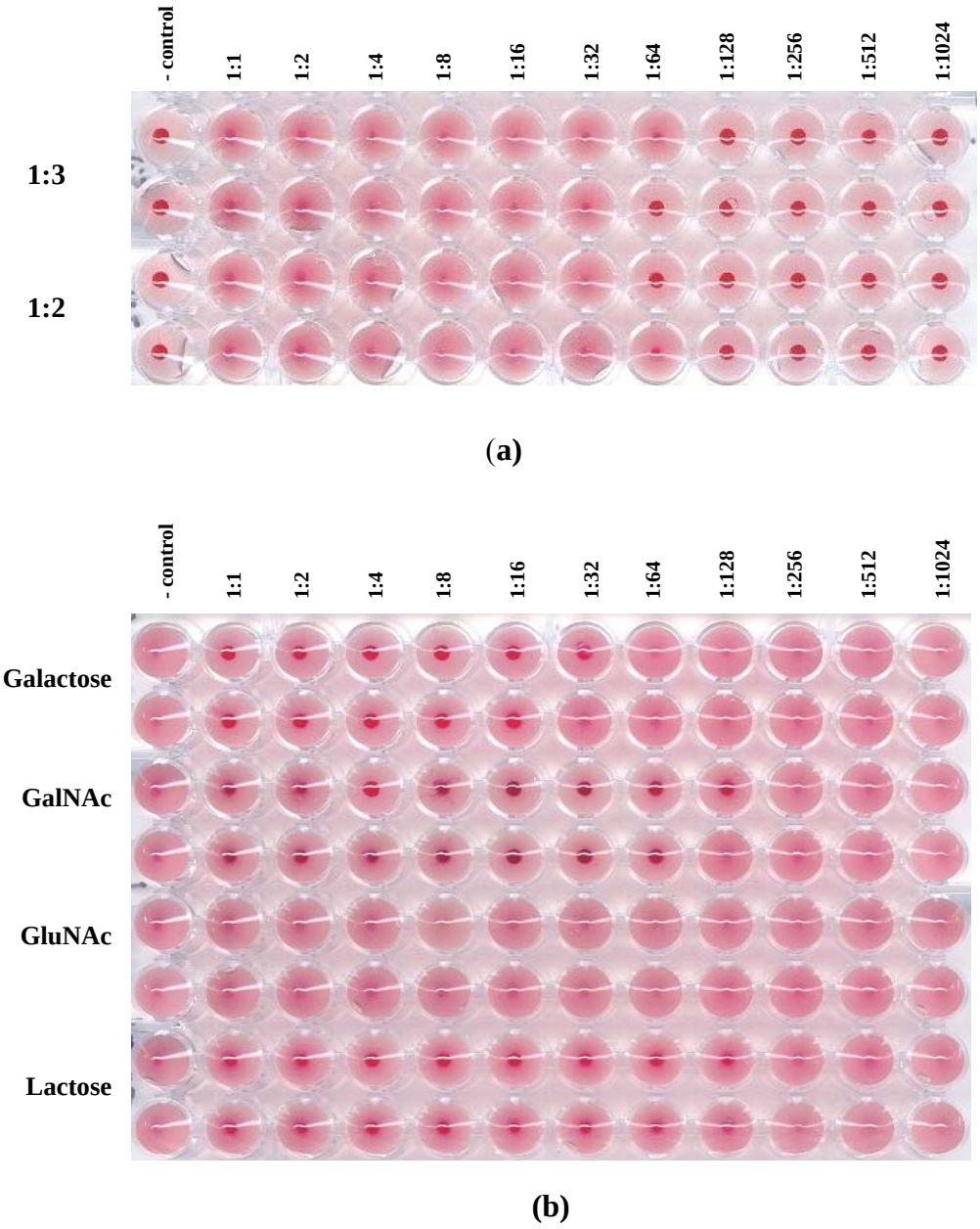

Figure 32. Sugar specificity of the purified lectin; standization of the purified lectin to obtain 16 HA units (a), and sugar inhibition assay against galactose (Gal), N-acetyl-D-galactosamine (GalNAc), N-acetyl-D glucosamine (GluNAc), and lactose (b).

4.4.6 Heat stability and pH stability

When tested SCL for heat stability, the activity started to decrease at 55ºC for 30 min test and was completely lost all their activity at 65ºC for 30 min test (Figure 33). Results from hemagglutination assay, the lectin was not markedly effected by pH, with maximum activity being retained at pH 5.0 to 9.0 (Figure 34). The pH gradient test showed that SCL had the highest stability at pH 8.0-9.0. Since Mg^{2+} and Ca^{2+} were presented in the extraction buffer and running buffer for affinity column, it was assumed that the lectin required both Mg^{2+} and Ca^{2+}at 10 mM for their activity. These results demonstrated that SCL was not heat-stable but stable to pH change over a wide range.

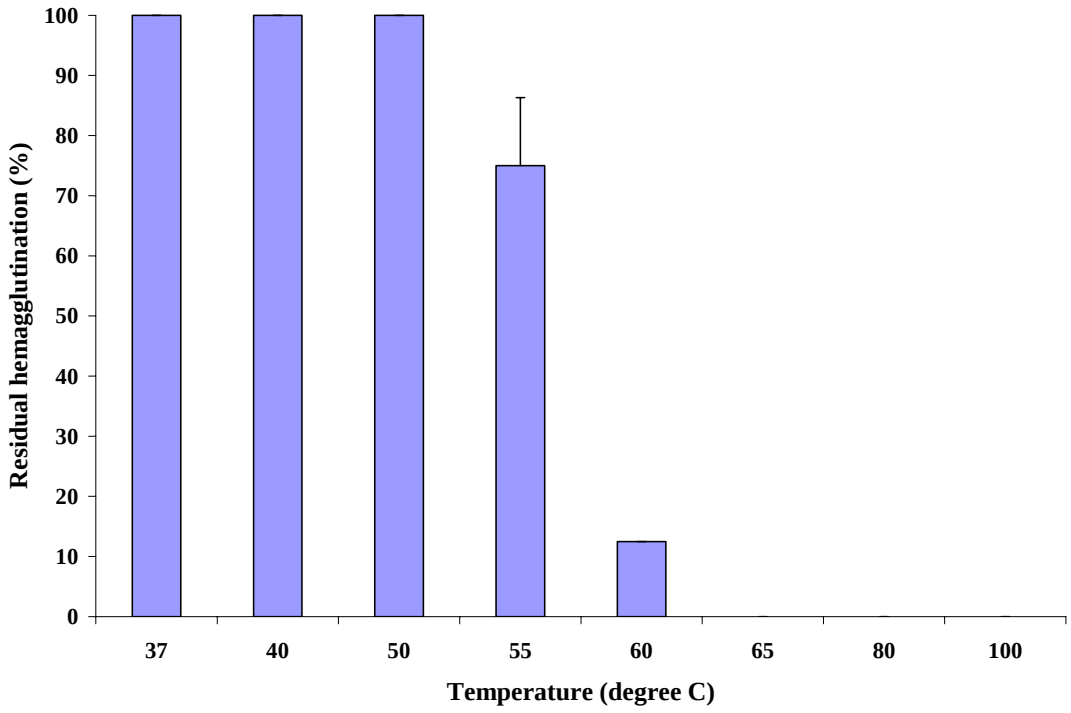

Figure 33. Heat stability of SCL. Hundred percentage of activity corresponded to a titer of 8.

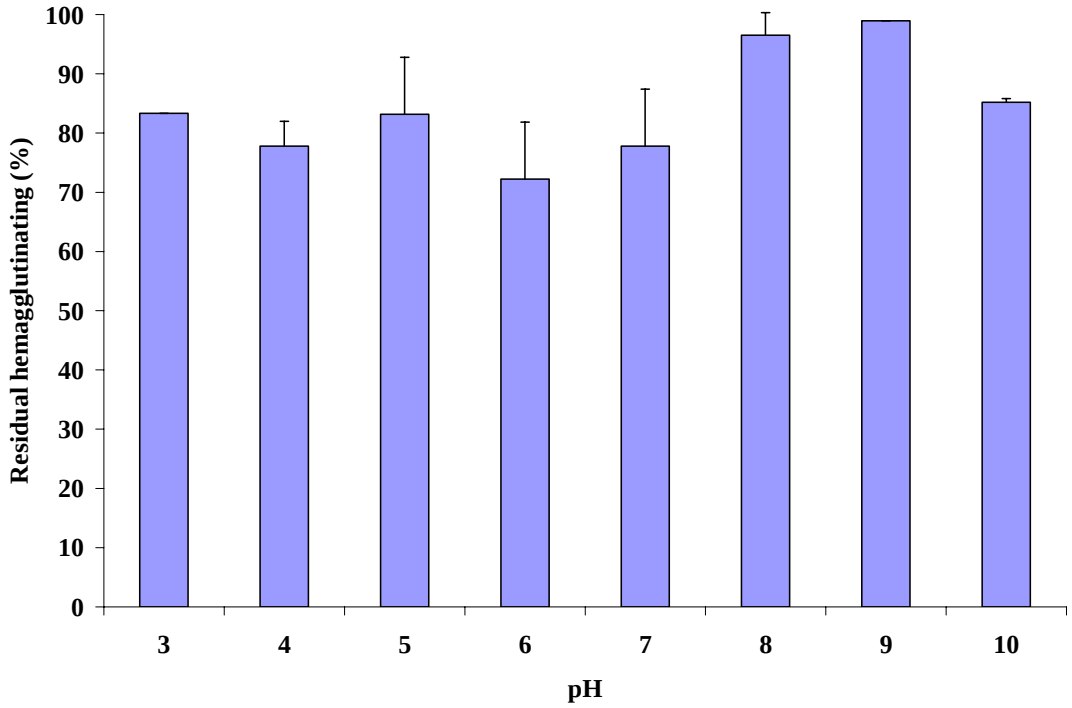

Figure 34. The pH stability of SCL. Hundred percentage of activity corresponded to a titer of 8.

4.4.7 Amino acid sequence analysis

After the SCL band from the tricine SDS-PAGE was blotted onto a polyvinyllidene difluoride membrane and applied to the automated sequencer, no amino acid peaks were obtained. It indicated clearly that this SCL has a blocked N-terminus. Then a tryptic digestion of the SCL was also attempted in order to determine the partial amino acid sequence. From the fractionated protein by reverse phase HPLC, some selected peptides were sequenced (Figure 35).

Although the N-terminus of SCL was blocked as the other mushrooms of *Agrocybe cylindracea* (Wang *et al.*, 2001), *Grifola frondasa* (Kawagishi *et al.*, 1990), and *Mycoleptodonoides aitchisonii* (Kawagishi *et al.*, 2001), some internal amino acid

sequences of tryptic peptides from SCL were determined. Then the SCL partial sequences were submitted to the protein database (the NCBI Blast database and FASTA search). Some sequence similarity with other lectins and other proteins were observed (Appendix G). The results from the NCBI Blast database demonstrated that this lectin had the highest percentage similarity at 66% with the agglutinin of mushroom, *Marasmius oreades*, following by 53% similarity with Ricin B-related lectin which was purified from *Polyporus squamosus*, 46% similarity with putative protein from small plant *Arabidopsis thaliana*, and lastly 35% similarity with 29-kDa galactose-binding lectin from *Lumbricus terrestris*. These confirmed specificity results of this lectin with galactose, and suggested that these lectins might be encoded by a family of related gene. Moreover the results from FASTA database (Figure 36 and more detail in Appendix G) demonstrated that the lectin has similarity with an envelop protein of a virus that have been reported as another class of lectins found in virus.

Peptide A: I Q G C V G G R D E

Peptide B: G T P I I G W D Y X E X

Peptide C: T F L A D I K P G X

Figure 35. Three peptide sequences of the purified *Schizophyllum commune* lectin obtained from a tryptic digestion.

2-1-3 GTPIIGWDYXEX IQGCVGGRDE TFLADIKPGX

>gi|18476512|gb|AAL47680.1| agglutinin [Marasmius oreades]
Length = 293
Score = 21.9 bits (45), Expect = 8.5
Identities = 6/9 (66%), Positives = 8/9 (88%)

```
Query: 1  GTPIIGWDY 9
          GTPI+GW +
Sbjct: 29 GTPIVGWQF 37
```

3-1-2 TFLADIKPGX IQGCVGGRDE GTPIIGWDYXEX

>gi|34915974|dbj|BAC87876.1| Ricin B-related lectin [Polyporus squamosus]
Length = 292
Score = 22.3 bits (46), Expect = 6.5
Identities = 7/13 (53%), Positives = 8/13 (61%)

```
Query: 16 GGRDEGTPIIGWD 28
          G    GTP+I WD
Sbjct: 27 GSGQNGTPVIAWD 39
```

2-3-1 GTPIIGWDYXEX TFLADIKPGX IQGCVGGRDE

>gi|7263614|emb|CAB81580.1| putative protein [Arabidopsis thaliana]
Length = 1113

Score = 22.3 bits (46), Expect = 6.5
Identities = 7/15 (46%), Positives = 11/15 (73%)

```
Query: 17  DIKPGXIQGCVGGRD 31
           D++PG ++G VG  D
Sbjct: 814 DVEPGIVEGSVGTED 828
```

Figure 36. Sequence similarity of the tryptic peptides of *Schizophyllum commune* lectin with other lectins from the NCBI database.

Although crude extract of SCL showed some antifungal activities against *Aspergillus niger* ATCC 6275, no more activity was further observed in the purified lectin. However, cytotoxic activity of SCL was tested against human epidermoid and cervical carcinoma cell lines. It was exhibited that SCL had high specificity against human epidermoid carcinoma cells rather than human cervical carcinoma cells with 20 μg/ml of the lectin.

In conclusion, a simple affinity chromatography protocol could successfully be used in the lectin purification for the purpose of crystallization.

4.4.8 Crystallization of the mushroom lectin

Protein structure can be determined by either Nuclear Magnetic Resonance (NMR) and X-ray crystallography techniques. However, an X-ray crystal structure can provide more precise values of atomic coordinates than does NMR (Lesk, 2001). To produce high quality lectin crystals, at least 5 mg of pure soluble lectin were needed. SDS-PAGE was also used to examine the purity of the soluble protein and aggregation before crystallization was attempted (Haward and Brown, 2002).

For lectin crystallization in this study, both vapor diffusion hanging drop and sitting drop techniques were performed. Results from manually prepared hanging drop with the sparse matrix showed protein ribbons which implied that the condition set up was close to the right condition of the lectin crystal growth. However, some parameters, particularly protein concentration, ammonium sulphate salt concentration, and pH of crystallization solution, must be optimized.

For the sitting drop experiments which were set up using a crystallization robot, lectin crystals could be observed under a microscope after 1 week. By using crystallization-screening reagent kits, JBScreen HTS I showed much more promise to establish the optimal condition for SCL crystal growth compared to JBScreen HTS II. By using JBScreen HTS I, different shapes of lectin crystals were grown under different conditions and appeared within one to two weeks (Figures 37). Crystal screen-scoring sheet from the observation in a crystallization experiment from JBScreen HTS I are summarized in Appendix H. Clear drops indicated either the relative supersaturation of the lectin solution and crystallization reagent was too low, or the drop had not yet completed equilibration. While drops containing precipitate indicated that either the relative supersaturation of the lectin sample or crystallization

reagent was too high, the sample had denatured, or the sample was heterogeneous. To reduce the relative supersaturation, it was suggested that the sample must be diluted in at lease two-fold. However, the precipitate should not be ignored because it was possible to obtain crystals from the precipitate by further seeding technique. The examination of the drops containing precipitate under polarizing optics could help differentiate precipitate from microcrystalline material (Hampton Research, 2003). However, the typical plate-like development of the crystals were obtained from this study when 18% PEG 8000, 0.1 M Na HEPES pH 7.5, 0.2M Ca acetate or 15% PEG 8000 (G9), 0.1 M Na MES pH 6.5, 0.2 M Na acetate (G10) were used as reservoir buffer (Figure 38). The crystals were grown up to 0.1 mm in linear dimensions.

This sitting drop experiment prepared by crystallization robot was very useful since only a half microliter of sample was needed for each drop. However, some disadvantages of the crystallization were observed. As shown in Figure 37 drop condition E06, it was noticed that the drop was not completed and was caused by unclean injection tube when a previous sticky solution was used and difficult to clean off.

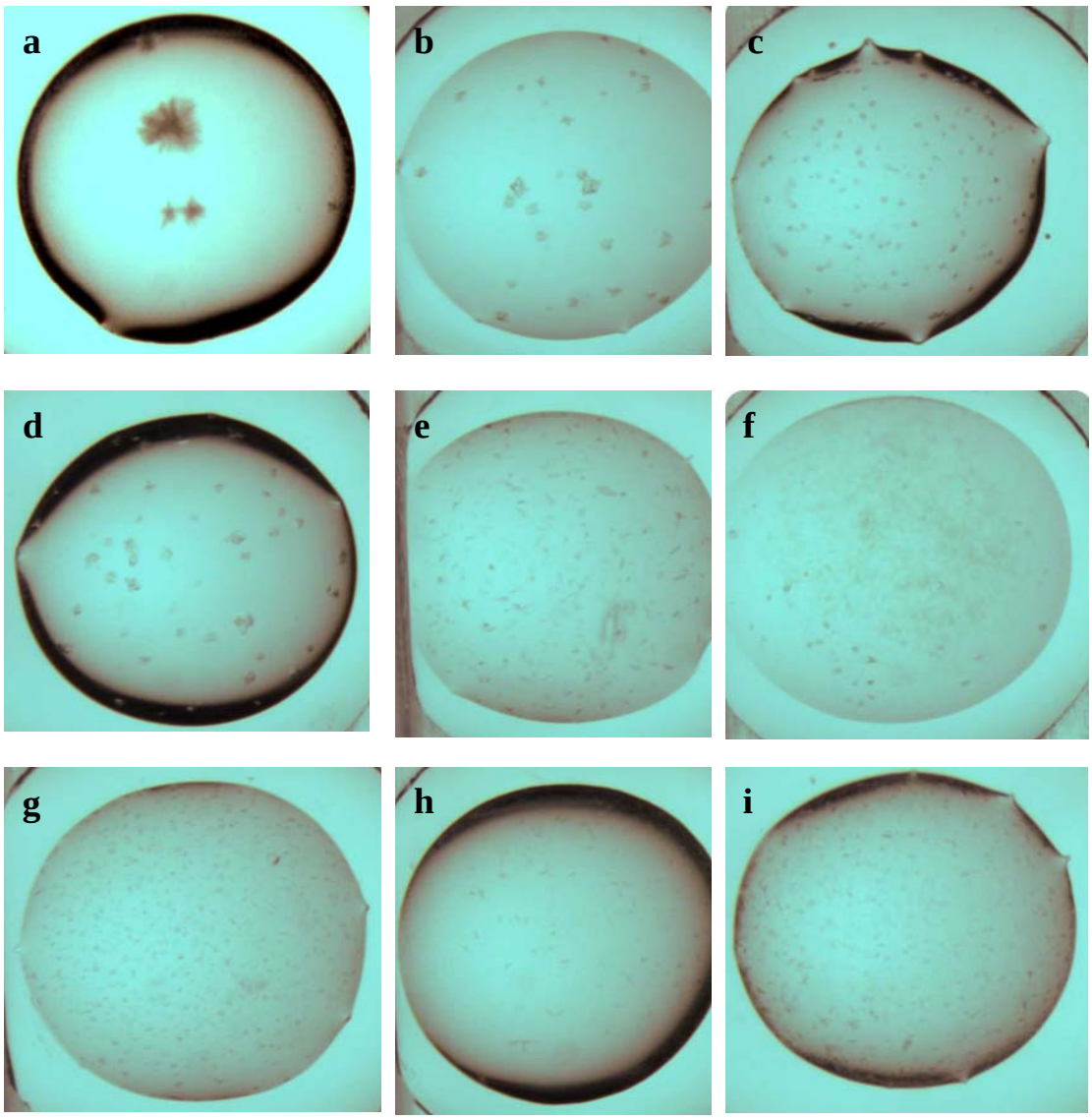

Figure 37. The *Schizophyllum commune* lectin crystals grown in sitting drops prepared using crystallization screening JBScreen HTS I. Conditions: a-x = A12, B03, B05, B06, C05, C06, C08, C12, D01, D09, D10, E01, E02, E03, E04, E05, E06, F04, F05, G07, G08, C09, G10, and G11 respectively after two weeks of incubation at 4ºC.

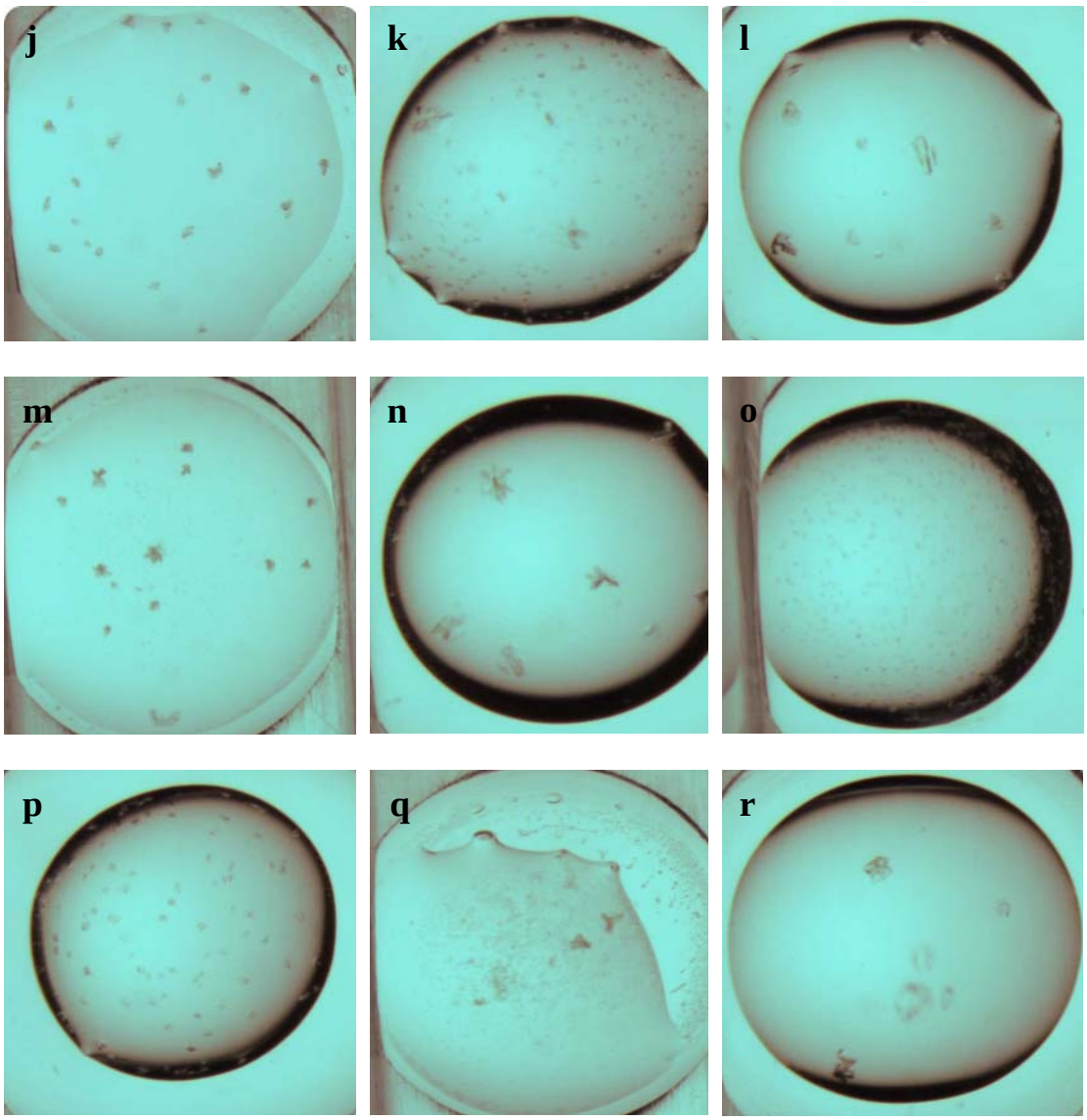

Figure 37. (Continued)

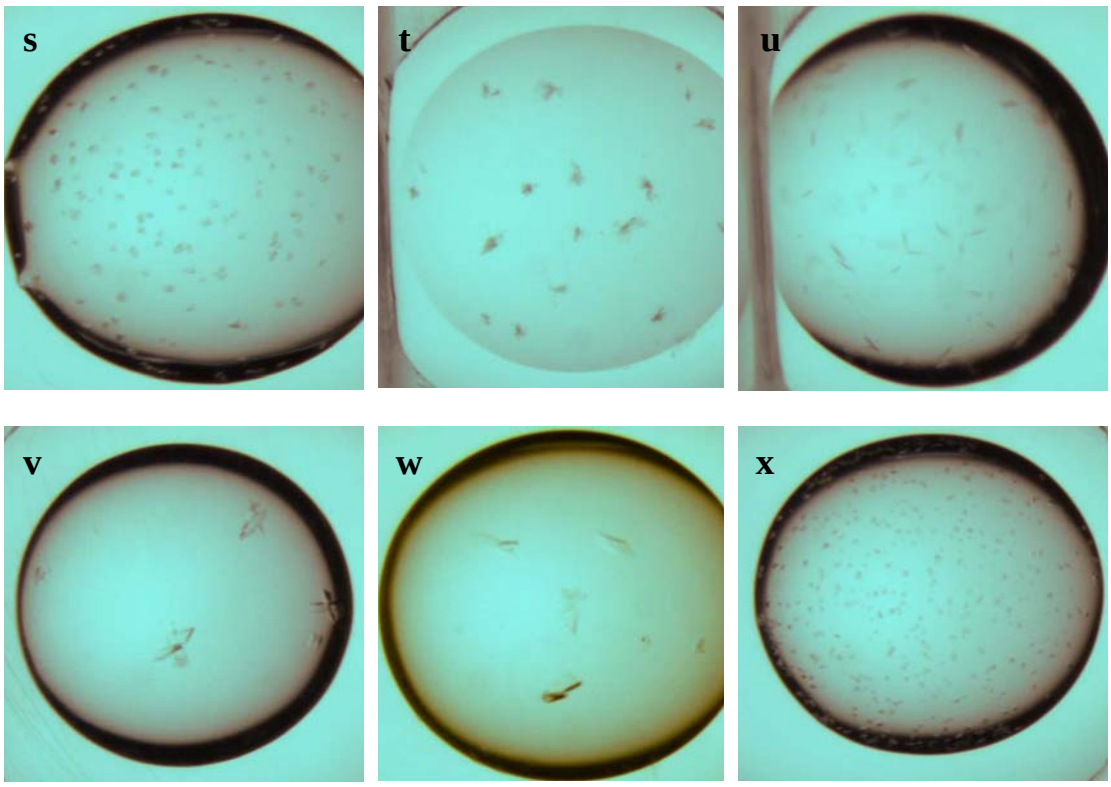

Figure 37. (Continued)

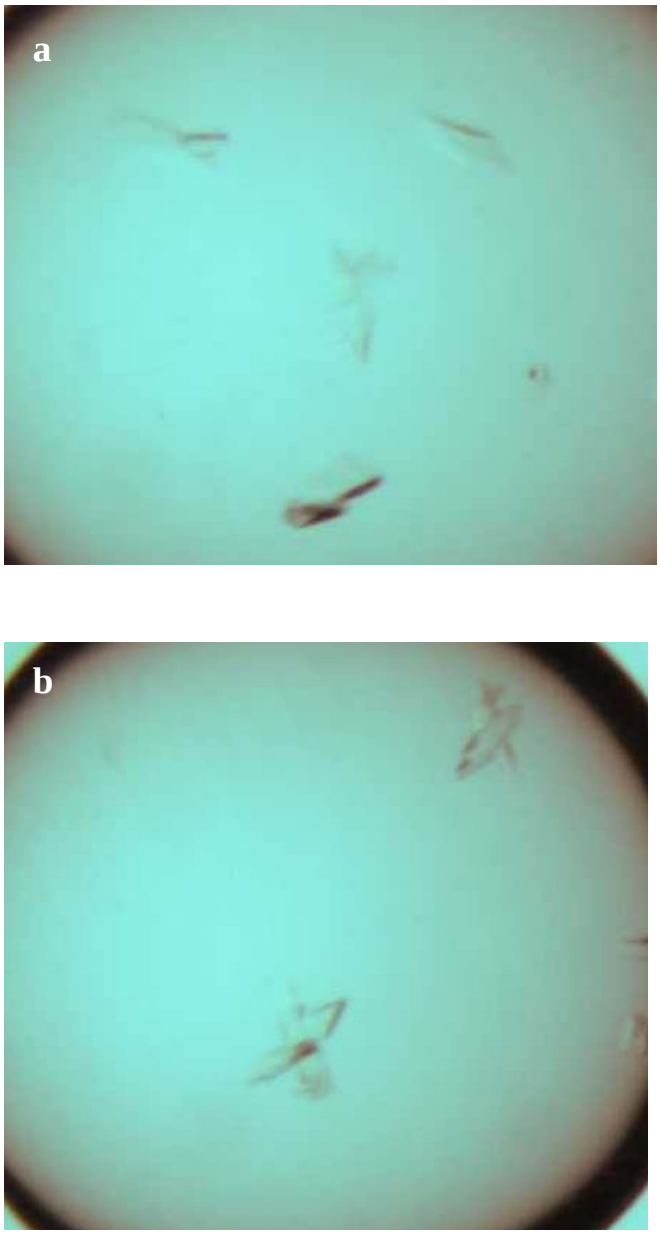

Figure 38. Crystals of *Schizophyllum commune* lectins grown in the droplets buffer solution of 15% PEG 8000, 0.1 M Na MES pH 6.5, 0.2 M Na acetate(a), and 18% PEG 8000, 0.1 M Na HEPES pH 7.5, 0.2 M Ca acetate (b) after 3 week incubation at 4ºC.

4.4.9 Preliminary analysis of the mushroom lectin structure

After the mushroom lectin crystals were successfully grown to obtain approximately 0.1 mm in the longest dimension that sufficient volume of crystal lattice could be exposed to the beam (Martin and Smyth, 2000), X-ray diffraction analysis could be performed to elucidated whether they are macromolecules or salts.

Although, nowadays most macromolecular X-ray diffraction experiments using X-ray radiation produced by a rotating anode X-ray generator, the X-ray diffraction experiments in this study were carried out using X-ray radiation produced by electrons losing angular momentum while orbiting in the high energy synchrotron storage rings at station 14.1 located at the Synchrotron Radiation Source, Daresbury Laboratory, U.K. The use of synchrotron radiation source is much more valuable than the rotating anode generators since higher intensity allows micro crystals to be used and the ability to select the wavelength of the X-ray radiation emitted has also allowed extension of the tractable resolution for an increasing number of protein structures to close to atomic resolution roughly 1.2 Å or better (Rhodes, 2000; Haward and Brown, 2002).

To increase the lifetime of some protein crystals by reducing radiation damage during data collection, it has been suggested that cooling may increase the internal order of parts of the protein which are mobile at room temperature (Giege and Ducruix, 1992). By cryo-crystallography, these crystals were mounted frozen in a small loop in a stream of liquid nitrogen onto a goniometer and X-ray diffraction was performed at low temperature, polyethylene glycol that was readily in the mother liquor was considered that beneficial act as cryoprotectants to help preventing of the ice formation (Rhodes, 1993). Thus preparation of crystals for cryocrystallography by

briefly placing them in a cryoprotected mother liquor for 5-15 sec to wash off the old mother liquor before they are immediately frozen was completely ignored. Thus some ice formation could be observed during this process. That might be the cause why lower resolutions were obtained than expected. However the X-ray diffraction data was extended to a maximum resolution of 3.8 Å (Figure 39). This suggested that soaking crystals before mounting for cryo-crystallography has significantly effects on the resolution of the mushroom lectin crystals. From the X-ray diffraction pattern, to reach higher resolution, crystal size and quality must be improved. More protein crystallization trials must be performed and the preliminary conditions should be finely optimized, and X-ray diffraction experiments must be carried out with care especially during the mounting process.

Because a crystal is made up of atoms or molecules that form a pattern that is repeated periodically in three dimensions. The unit cell can be described by three lengths, termed a, b, and c, representing the edges or axes of the unit cell and three angles, termed α, β, and γ, representing the angles between two of the corresponding axes (Howard and Brown, 2002). Based on the unit cell parameters, roughly from the diffraction pattern (Figure 39), a monoclinic crystal system with lattice symmetry 2/m and unit cell constraints $\alpha = \gamma = 90^{\circ}$ and $\beta > 90^{\circ}$ could be expected.

This work was successfully proved that the tropical mushroom can provide lectin crystals for structural biological analysis. This can also promote natural drug discovery investigations since the lectin crystals could be obtained at the first batch of sitting drop technique experiment, and the lectin crystals were proved to be real protein crystals. They were not salt crystals.

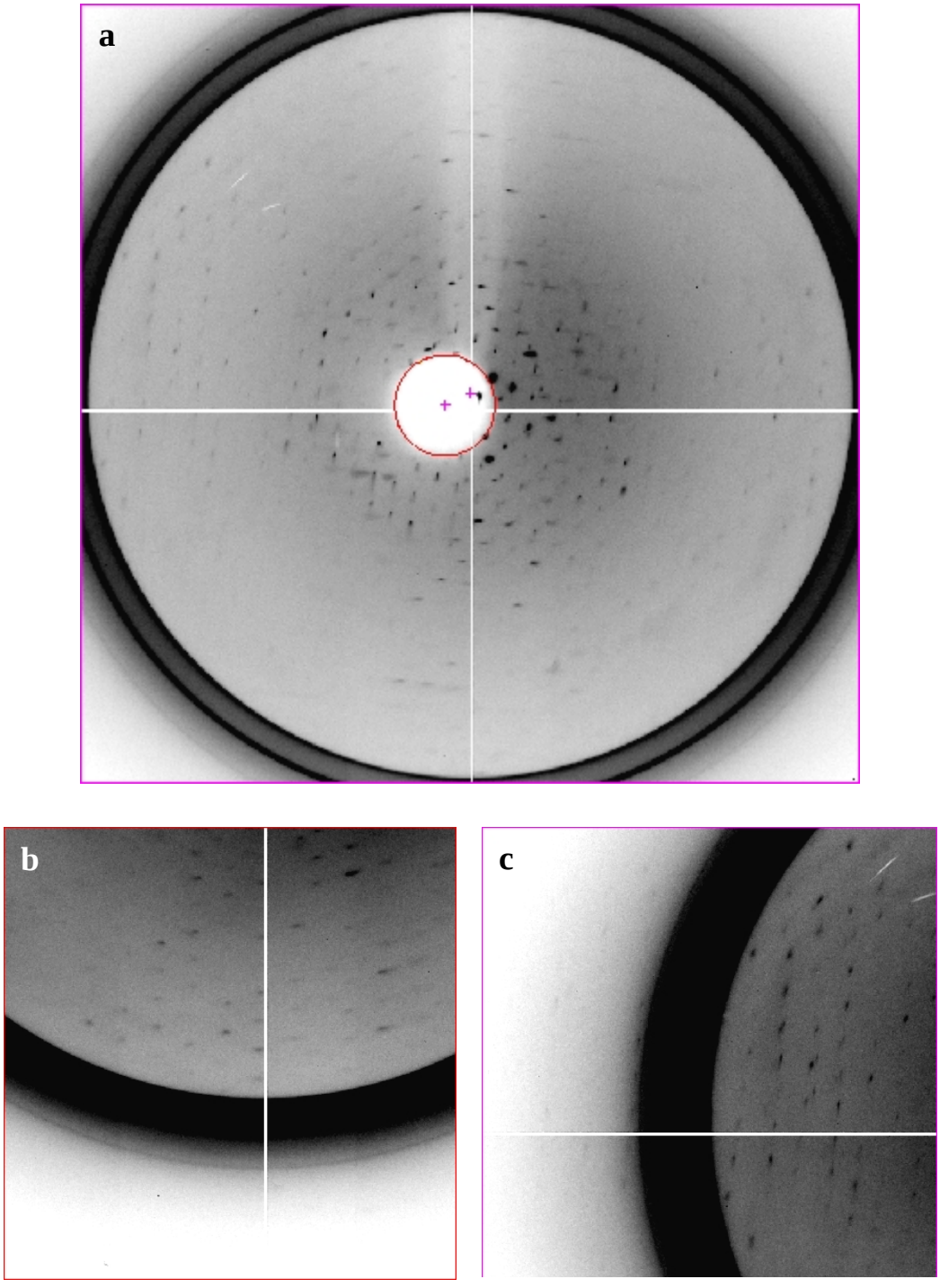

Figure 39. Diffraction pattern from a crystal of the *Schizophyllum commune* lectin; a, whole pattern; b, bottom view; and c, right side view.

CHAPTER V

CONCLUSION AND FUTURE PERSPECTIVE

Throughout this study, mushroom surveys were conducted during the rainy season August-October, 2002 and June-August, 2003 from a variety of natural habitats, natural forests, and local markets in the Northeastern, Central, and Western Thailand. A total of two hundred and eighteen mushroom specimens from Nakhon Ratchasima, Burirum, Nakhon Pathom, and Kanchanaburi Provinces were collected and identified by using both macroscopic and microscopic characterization methods. Totally 48 mushroom genera of 23 families were recorded. In this study, the most frequently encountered basidiomycorta were from *Tricholomataceae* followed by *Russulaceae, Agaricaceae, Plurotaceae*, *Amanitaceae,* and *Boletaceae*.

According to their carbohydrate binding specificity of the lectin, hemagglutination assay was investigated to determine the lectin accumulation in the mushroom specimens. The preliminary screening of mushroom lectins showed clearly that a wide variety of tropical mushrooms in Thailand accumulated lectins in their fruit bodies. The lectins could be simply extracted and basically tested for their activity. More than 60% of mushroom extracts were found to predominantly perform hemagglutinating for rat red blood cells (RBCs).

The high incidence of lectin accumulations was observed in mushrooms, which belonged to genera *Amanita, Macrocybe, Macrolepiota, Lycoperdon, Termitomyces,* and *Volvariella*. The hemolytic activity and partial hemolysis of mushroom extracts

against human and animal erythrocytes were also detected at approximately 40%. The mushrooms, which accumulated high hemolysin belonged to genera *Amanita*, *Entoloderma, Macrolepiota,* and *Lycoperdon.* This information on hemolytic activity and partial hemolysis is useful for the medical research application such as hemolysin production.

This investigation showed that 60% of tropical mushrooms (218 specimens) had lectin accumulation. This percentage is close to the value found for a previous lectin survey in Great Britain of 65% from 72 British mushroom species (Anstee, 1972 quoted in Pemberton, 1994). However, it showed higher percentages than that of the more recent work of Pemberton (1994) who found that agglutinins accumulated at 50% for the 403 species and was also higher than lectin studies made in France and Germany which demonstrated that agglutinin contained in the fungi at thirty percent yield from over 600 species and 20% from 293 species, respectively (Guillot *et al*., 1983; Seeger and Weildmann, 1972). This variation might be due to the different serological screening method and varieties of animal RBCs.

Mushroom mycelia from genera *Russula* and *Amanita* were investigated, and preliminary tested for their lectin accumulation. Even some of them were successfully cultured but a very low content of lectin was determined and difficulty in culture isolation. Therefore, only lectin accumulation in fruit bodies was determined in this study.

The antimicrobial activity of some selected mushroom lectins was tested using the agar disc diffusion assay. The crude extracts of *Microporus* showed considerable activity against the Gram-negative bacterium, *Bacillus cereus* ATCC 6633 and the fungus, *Aspergillus niger* ATCC 6275. The extract of *Termitomyces* had inhibitory

activity with the Gram-positive bacterium, *Staphylococcus aureus* ATCC 29213. The *Stereum* extract showed positive results against both *Staphylococcus aureus* ATCC 29213 and the yeast *Saccharomyces cerevisiae* ATCC 26108. The mushroom extract from *Schizophyllum commune* Fr.: Fr showed moderately inhibition zone with *Aspergillus niger* ATCC 6275.

Although low antimicrobial activity could be obtained from these mushroom lectins, the information was still promising important for future research because nowadays extraction of bioactive compounds directly from their natural source is not the only way for the investigation. If the structure of the bioactive compound was elucidated, using knowledge on recombinant DNA technology could possibly produce a synthetic compound. And since antimicrobial-resistant organisms have been the major problem in medical treatment, searching for new antimicrobial compounds are still is interested.

From total 60 selected mushroom extracts, more than 90% and 95% of them exerted some cytotoxic action on human epidermoid carcinomas and human cervical carcinomas. Trend of cytotoxic activity against both cancer cell types was in the same direction. The highest antiproliferation activity on human epidermal carcinomas was observed on mushroom genera *Amanita* and following by *Strobilomyces*, *Lentinus*, *Russula*, and *Cantharellus* while extracts which displayed highest antiproliferation activities on human cervical carcinomas were obtained from *Lentinus*, *Amanita*, *Macrolepiota*, *Marasmius*, and *Russula*. Most of these mushrooms are edible. So this work demonstrated that traditional knowledge about medicinal mushroom of local people is reliable. Since the well known of anticancer mushroom genus *Lentinus* was utilized (Seewapong, 2003), their efficacy could be confirmed by this work. It was

also interesting that the popular edible mushroom, *Cantharellus* that are available in high quantity in the local area of Northeastern, Thailand showed efficiently cytotoxicity against human epidermoid carcinomas. It is good for drug development since epidermoid carcinoma turn to be a major cause of cancer patient in Thailand right now.

From reasons of mushroom availability, biological activities, and a difficulty of purification, the edible mushroom, *Schizophyllum commune* with moderate antifugal and cytotoxic activity was finally selected for the purification and characterization of lectins. A lectin could be isolated by affinity chromatography on a mucin-Sepharose 4B column. Molecular weight of the purified lectin was 31 kDa. The lectin was glycoprotein, which showed specificity towards both galactose and N-acetylgalactosamine. Their lectin crystals could be attempted from crystallization by the sitting drop vapour-diffusion method with polyethylene glycol 8000 as precipitate. This lectin gave crystals, which gave an X-ray diffraction pattern to approximately 3.8 Å resolution. This work was successfully proved that the tropical mushrooms could be a good source for medicinal lectin investigation. Some tropical mushroom lectins could be possibly purified and crystallized. The crystal could be used in the X-ray crystallography that is beneficial for structural-based drug design in the future study.

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APPENDICES

APPENDIX A

CULTURE MEDIUM AND REAGENT PREPARATIONS

1. Buffer for mushroom lectin extraction

1.1 Phosphate buffer saline (PBS) for the mushroom lectin extraction to determine lectins accumulated in mushroom specimens

NaCl	8.00	g
KCl	0.20	g
Na_2HPO_4	1.15	g
KH_2PO_4	0.20	g
Sodium bisulpite (0.02 M)	22.80	g

Adjust pH to 7.4 with HCl and the volume to 1 l with D.W.

1.2 PBS for the extraction in the antimicrobial assay and purification of mushroom lectin

NaCl	8.00	g
KCl	0.20	g
Na_2HPO_4	1.15	g
KH_2PO_4	0.20	g
Benzamidine (1mM)	0.15	g
2-Mercaptoethanol (0.1%)	2.00	ml
Polyvinylpyrrolidone (PVPP) (1.5%)	7.50	g

NaN_3 0.02%	0.20	g

Adjust pH to 7.4 with HCl and the volume to 1 l with D.W.

2. Buffers and solutions for mushroom lectin purification

2.1 Running buffer for the mucin-Sepharose 4B affinity chromatography column

Tris (10 mM, prepared from 2M stock solution Tris-HCl, pH 8)	5.00	ml
$CaCl_2$ (1 mM)	0.15	g
$MgCl_2$ (1mM)	1.00	ml
NaN_3 (0.02%)	0.20	g

Adjust pH to 7.4 and the volume to 1 l with D.W.

2.2 Tris (2 M, pH 8.0)

Tris 2 M	242.28	g

Adjust pH to 7.4 and the volume to 1 l with D.W.

2.3 1, 3 diaminopropane (DAP), 20 mM

DAP (20 mM)	1.66	ml

Adjust the volume to 1 l with D.W.

2.4 Tris (1M, pH 8.0)

Tris (1 M)	121.14	g

Adjust pH to 7.0 and the volume to 1 l with D.W.

3. Buffers and solutions for SDS-PAGE

3.1 Resolving gel SDS-PAGE (17.5%)

Tris-HCl (1.5 M, pH 8.8)	3.75	ml
Bis-acrylamide (1%)	1.12	ml
D.W.	1.14	ml
SDS (20%)	0.15	ml
Acrylamide (30%)	8.75	ml
APS (10%)	0.10	g
TEMED	0.01	ml

3.2 Resolving gel SDS-PAGE (20%)

Tris-HCl (1.5 M, pH 8.8)	3.75	ml
Bis-acrylamide (1%)	0.98	ml
D.W.	0.03	ml
SDS (20%)	0.15	ml
Acrylamide (30%)	10.00	ml
APS (10%)	0.10	ml
TEMED	0.01	ml

3.3 Stacking gel SDS-PAGE (5%)

Tris-HCl (1.0 M, pH 6.8)	3.75	ml
Bis-acrylamide	3.90	ml
D.W.	4.00	ml
SDS (20%)	0.15	ml
Acrylamide (30%)	2.50	ml
Bromophenol blue (0.1%)	0.60	ml
APS (10%)	100.00	µl
TEMED	20.00	µl

3.4 Running buffer for SDS-PAGE

Glycine	14.40	g
Tris-base	3.03	g
SDS	0.50	g

Make up solution to 500 ml with D.W.

3.5 Destaining solution for Coomassie stain

Methanol	450.00	ml
Glacial acetic acid	100.00	ml

Make up solution to 1 l with D.W.

3.6 Staining solution with Coomassie Brilliant Blue

Coomassie Brilliant Blue R-250	2.00	g
Methanol	450.00	ml
Glacial acetic acid	100.00	ml

Make up solution to 1 l with D.W.

3.7 SDS-gel loading buffer

Tris-HCl (1.0 M, pH 6.8)	4.00	ml
SDS	1.00	g
2-mercaptoethanol	0.50	ml
Bromophenol blue (0.1%)	1.00	g
Glycerol	10.00	ml

Make up solution to 50 ml with D.W.

3.8 Tris-HCl (1.0 M, pH 6.8)

1.0 M Tris-HCl	121.14	g

Adjust pH 6.8 with HCl and the volume to 1 l with D.W.

4. Solution of 5% polyacrylamide native particle gel

4.1 TBE buffer 10X (1000 ml)

Tris Base (0.89 M)	107.70	g
Boric acid (0.89 M)	55.00	g
EDTA (disodium form) (25 mM)	8.40	g

4.2 Acrylamide solution for particle gel (30%)

Acrylamide	29.00	g
N,N,N,N Methylene bisacrylamide	1.00	g

4.3 Recipe for particle gel

Acrylamide solution (29:1) (30%)	8.30	ml
TBE 10X	5.00	ml
TEMED	0.10	ml
Ammonium persulphate (10%)	0.16	ml
D.W.	36.40	ml

4.4 Sample buffer

TBE buffer 10X	10.00	ml
Bromophenol blue (0.1%)	0.10	g
Glycerol (20%)	20.00	ml

Make up the volume to 100 ml with D.W.

5. Periodic Acid Schiff (PAS) staining of polyacrylamide

5.1 Fixation/destaining solution

Acetic acid	10.00	ml

Methanol	35.00	ml
D.W.	55.00	ml

5.2 Meta-bisulfite solution

Dissolve 0.2 g sodium meta-bisulfite ($Na_2S_2O_5$) in 100 ml of 5% (v/v) acetic acid (made fresh).

6. Tricine SDS-PAGE (for small protein and peptide separation)

6.1 Gel buffer

Tris-HCl (3.0 M, pH 8.45)	181.00	g
SDS (0.3%)	1.50	g

Make up the solution to 500 ml with D.W.

6.2 Acrylamide solution

Acrylamide (48%)	240.00	g
Methyline bis-acrylamide (1.5%)	7.00	g

Adjust the volume to 500 ml with D.W.

6.3 Glycerol solution

Glycerol (50%)	250.00	ml

Adjust the volume to 500 ml with D.W.

6.4 Running buffer for Tricine SDS-PAGE

Tris base (100 mM)	6.06	g
Tricine (100 mM)	8.96	g
SDS (0.1%)	0.50	g

Make up solution to 500 ml with D.W.

6.5 Resolving gel SDS-PAGE (12%)

Acrylamide solution	7.20	ml
Gel buffer	10.00	ml
Glycerol solution (50%)	6.70	ml
D.W.	4.10	ml
APS (10%)	0.10	ml
TEMED	0.01	ml

6.6 Stacking gel (4%)

Acrylamide solution	1.00	ml
Gel buffer	4.00	ml
D.W.	6.35	µl
Bromophenol blue (0.1%)	600.00	µl
APS (10%)	100.00	µl
TEMED	20.00	µl

7. Reagents for mushroom identification

7.1 Lactophenol

Lactic acid	20.00	ml
Phenol crystal	20.00	g
Glycerol	40.00	ml
D.W.	20.00	ml

Kept in the bottle (may add 0.05 g of cotton blue or methylene blue).

7.2 Melzer's reagent

Iodine	1.50	g

Potassium iodide	5.00	g
Chloral hydrate	100.00	g
D.W.	100.00	ml

Make up solution to 100 ml with D.W.

7.3 Ammonium hydroxide 10%

Dissolved 10 ml of ammonium hydroxide in D.W. and adjust to 100 ml final volume.

8. Culture media for mushroom culturing and antimicrobial activity test

8.1 Potato dextrose agar

Potato dextrose	4.00	g
Glucose	20.00	g
Agar	15.00	g

Final pH 5.6 ± 0.2 at room temperature

The compositions were suspended in 1 l of distilled or deionized water. Bring to the boil until dissolved completely. Sterilization was done by autoclaving for 15 min at 121°C.

8.2 Malt extract agar

Malt extract	30.00	g
Peptone	5.00	g
Agar	15.00	g

Final pH 5.4 ± 0.2 at room temperature

The compositions were suspended in 1 l of distilled or deionized water. Bring to the boil until dissolved completely. Sterilization was done by autoclaving for 15 min at 121°C.

8.3 Mueller-Hinton agar

Beef, dehydrated infusion form	300.00	g
Casien hydrolysate	17.50	g
Starch	1.50	g
Agar	15.00	g

Final pH 7.3 ± 0.2 at room temperature

The compositions were suspended in 1 liter of distilled or deionized water. Bring to the boil until dissolved completely. Sterilization was done by autoclaving for 15 min at 121°C.

8.4 Nutrient broth

‘Lab-Lemco’ powder	1.00	g
Yeast extract	2.00	g
Peptone	5.00	g
Sodium chloride	5.00	g

Final pH 7.4 ± 0.2 at room temperature

The composition was suspended in 1 l of distilled or deionized water. The solution was mixed well and distributed into final containers. Sterilized by autoclaving for 15 min at 121°C.

9. Reagents for protein blotting

9.1 Transfer buffer

Dissolved 2.91 g of Tris base, 1.46 g of glycine, 0.1875 g of SDS in 100 ml methanol then adjust volume to 500 ml with D.W.

9.2 Destaining solution

Methanol (50%)	50.00	ml

Make up solution to 500 ml with D.W.

9.3 Low acid stain solution

Coomassie blue R 250 (0.2%)	1.00	g
Methanol (40%)	200.00	ml
Acetic acid (1%)	5.00	ml

Adjust the volume to 500 ml with D.W.

APPENDIX B

TRICHLOROACETIC ACID (TCA) PRECIPITATION METHOD

Prepare samples for electrophoretic examination by carrying out a TCA precipitation according to Bollag *et al*. (1996).

1. Calculate the volume of sample, which would contain 30 μg of protein, pipette into an eppindorf tube.
2. Add TCA to a final concentration of 25% from a stock 100% TCA.
3. Stand the sample/TCA on ice for 30 min.
4. Spin a microfuge for 10 min at high speed (orientate the tubes with the hinge outwards).
5. Suck off the supernatant.
6. Replace with 0.5 ml of acid acetone (0.1% HCl in acetone).
7. Repeat steps 4 and 5.
8. Replace with 0.5 ml of acetone.
9. Spin a microfuge for 10 min at high speed (orientate the tubes as before).
10. Resuspend the pellet in 10 μl of upper buffer (1.0 M Tris-HCl, pH 6.8), then add an equal volume of sample buffer.

APPENDIX C

AMMONIUM SULPHATE PRECIPITATION TABLE

INITIAL	10	20	25	30	35	40	45	50	55	60	65	70	75	80	90	100
0	56	114	144	176	209	243	277	313	351	390	430	472	516	561	662	767
10		57	86	118	150	183	216	251	288	326	365	406	449	494	592	694
20			29	59	91	123	155	189	225	262	300	340	382	424	520	619
25				30	61	93	125	158	193	230	267	307	348	390	485	583
30					30	62	94	127	162	198	235	273	314	356	449	546
35						31	63	94	129	164	200	238	278	319	411	506
40							31	63	97	132	168	205	245	285	375	469
45								32	65	99	134	171	210	250	339	431
50									33	66	101	137	176	214	302	392
55										33	67	103	141	179	264	353
60											34	69	105	143	227	314
65												34	70	107	190	272
70													35	72	153	237
75														36	115	198
80															77	157
90																79

APPENDIX D

COLLECTION OF MUSHROOM SPECIMENS

Table 11. List of all macrofungi collection during September-October 2002 and June-August 2003.

No.	Code no.	Fungal species	Location[1]	No. of specimen	Collection date
1	P001	*Auricularia polytricha* (Mont.) Sacc.	NR1	>15	30/8/2002
2	P002	*Lentinula squarrosulus* Mont.	NR1	>20	30/8/2002
3	P003	*Macrolepiota dolichaula* (Berk. & Br.) Pegler & Ray.	NR1	3-5	30/8/2002
4	P004	*Pleurotus* sp. (Hungarian)	NR1	>10	30/8/2002
5	P005	*Termitomyces* sp.	NR1	>10	30/8/2002
6	P006	*Lentinula edodes* (Berk.) Pegler	NR1	>15	30/8/2002
7	P007	*Agrocybe cylindracea* (DC.: Fr.) Maire	NR1	>15	30/8/2002
8	P008	*Pleurotus* sp. (Butan)	NR1	>20	30/8/2002
9	P009	*Pleurotus cystidiosus* O.K. Miller	NR1	>20	30/8/2002
10	P010	*Flammulina velutipes* (Fr.) P. Karst	NR1	>100	30/8/2002
11	P011	*Macrocybe* sp.	NR1	>15	30/8/2002
12	P012	*Lentinula squarrosulus* Mont.	NR5	>15	1/9/2002
13	P013	*Volvariella volvacea* (Bull.: Fr.) Sing.	NR5	>25	1/9/2002
14	P014	*Lentinula* sp.	NR5	>15	1/9/2002
15	P015	*Pleurotus sajor-caju* (Fr.) Sing.	NR5	>15	1/9/2002
16	P016	*Auricularia polyticha* (Mont.) Sacc.	NR1	>10	1/9/2002
17	P017	*Pleurotus cornucopiae* (Paul: Pers.) Rolland	NR1	>15	1/9/2002
18	P018	*Lentinus polychrous* Lev.	NR1	>10	1/9/2002
19	P019	*Macrocybe crassum*	NR5	>10	1/9/2002
20	P020	*Termitomyces* sp.	NR1	5-10	3/9/2002
21	P022	*Leucocoprinus* sp.	NR1	7	3/9/2002
22	P023	*Lepiota* sp.	NR1	>15	3/9/2002
23	P025	*Laccaria* sp.	NR1	>15	3/9/2002
24	P026	*Lepiota* sp.	NR1	>10	3/9/2002
25	P028	*Lentinus squarrosulus* Mont.	NR1	8	3/9/2002
26	P029	*Leucocoprinus* sp.	NR1	>10	3/9/2002
27	P030	*Lepiota* sp.	NR1	10-15	3/9/2002

Table 11. (Continued)

No.	Code no.	Fungal species	Location[1]	No. of specimen	Collection date
28	P032	*Lactarius* sp.	NR1	2	3/9/2002
29	P034	*Leucocoprinus* sp.	NR1	5-10	5/9/2002
30	P035	*Pleurotus cornucopiae* (Paul: Pers.) Rolland	NR1	>20	6/9/2002
31	P036	*Pleurotus djamor* (Fr.) Boedijn	NR1	>20	6/9/2002
32	P037	*Lepiota* sp.	NR1	>10	7/9/2002
33	P041	*Cantharellus minor* Peck.	NR1	>10	6/9/2002
34	P042	*Amanita* sp.	NR5	>10	7/9/2002
35	P043	*Amanita* sp.	NR5	>10	7/9/2002
36	P049	*Tylopilus* sp.	NR1	5-10	7/9/2002
37	P054	*Amanita* sp.	BL1	5-10	7/9/2002
38	P065	*Termitomyces* sp.	BL1	>10	10/9/2002
39	P066	*Russula* cf. *cyanoxantha* (Schaeff: Schw.) Fr.	BL1	>15	10/9/2002
40	P067	*Termitomyces* sp.	BL1	>10	10/9/2002
41	P068	*Termitomyces* sp.	BL1	>15	10/9/2002
42	P069	*Amanita* sp.	BL1	>10	10/9/2002
43	P070	*Russula* sp.	BL1	10-15	10/9/2002
44	P071	*Russula* sect. *nigricantae* Fr.	BL1	>10	10/9/2002
45	P072	*Russula alboareolata* Hongo.	BL1	>10	10/9/2002
46	P074	*Russula* sp.	BL1	>20	10/9/2002
47	P075	*Russula* sp.	BL1	>20	10/9/2002
48	P076	*Russula* sp.	BL1	>10	10/9/2002
49	P077	*Amanita* sp.	BL1	>15	10/9/2002
50	P078	*Cantharellus minor* Peck.	BL1	10-20	10/9/2002
51	P087	*Agrocybe cylindracea* (DC.: Fr.) Maire	NR1	10-15	18/9/2002
52	P089	*Lentinus* sp.	NR5	>15	18/9/2002
53	P090	*Pycnoporus* sp.	NR5	>15	18/9/2002
54	P092	*Pleurotus sajor-caju* (Fr.) Sing.	NR5	>10	18/9/2002
55	P093	*Leucocoprinus* cf. *birnbaumii* (Corda) Singer	NR1	5-10	18/9/2002

Table 11. (Continued)

No.	Code no.	Fungal species	Location[1]	No. of specimen	Collection date
56	P094	*Leucocoprinus* sp.	NR1	5-10	18/9/2002
57	P095	*Macrolepiota* sp.	NR1	>10	18/9/2002
58	P096	*Lycoperdon* sp.	NR1	10-15	18/9/2002
59	P097	*Amanita* sp.	NR1	>5	18/9/2002
60	P098	*Craterellus* sp.	NR1	>30	18/9/2002
61	P099	*Marasmius* sp.	NR1	>50	18/9/2002
62	P100	*Laccaria* sp.	KB	>40	18/9/2002
63	P101	*Termitomyces* sp.	KB	>30	18/9/2002
64	P102	*Pleurotus djamor* (Fr.) Boedijn	NP	>20	25/9/2002
65	P103	*Pleurotus citrinopileatus* Sing.	NP	>20	25/9/2002
66	P104	*Pleurotus* sp. (Butan)	NP	>20	25/9/2002
67	P105	*Schizophyllum commune* Fr.: Fr.	NP	>70	25/9/2002
68	P106	*Lentinula edodes* (Berk.) Pegler	NP	>10	25/9/2002
69	P107	*Lentinula polychrous* Lev.	NP	>10	25/9/2002
70	P108	*Auricularia polytricha* (Mont.) Sacc.	NP	>15	25/9/2002
71	P109	*Lentinus* sp.	NP	>10	25/9/2002
72	P110	*Pleurotus ostreatus* (Jacq.: Fr.) P. Kumm.	NP	>15	25/9/2002
73	P111	*Auricularia* sp.	NP	>10	25/9/2002
74	P112	*Pleurotus* sp. (Hungarian)	NP	>15	25/9/2002
75	P113	*Macrolepiota* sp.	NR1	17	30/9/2002
76	P114	*Lentinus polychrous* Lev.	NR5	>20	30/9/2002
77	P115	*Laccaria* sp.	NR5	14	30/9/2002
78	P116	*Termitomyces* sp.	NR5	5	30/9/2002
79	P117	*Termitomyces* sp.	NR5	7	30/9/2002
80	P118	*Russula* sp.	NR5	>15	30/9/2002
81	P119	*Russula* sp.	NR5	>10	30/9/2002
82	P120	*Russula* sp.	BL3	>20	8/10/2002
83	P121	*Flammulina velutipes* (Fr.) P. Karst.	NR5	>50	8/10/2002

Table 11. (Continued)

No.	Code no.	Fungal species	Location[1]	No. of specimen	Collection date
84	P122	*Pleurotus sajor-caju* (Fr.) Sing.	NR5	>15	8/10/2002
85	P123	*Laccaria* sp.	BL4	>20	8/10/2002
86	P124	*Termitomyces* sp.	NR1	15	8/10/2002
87	P125	*Termitomyces* sp.	NR1	8	8/10/2002
88	P126	*Flammulina velutipes* (Fr.) P. Karst	NR1	>1000	8/10/2002
89	P127	*Flammulina velutipes* (Fr.) P. Karst	NR1	>1000	8/10/2002
90	P128	*Termitomyces* sp.	NR4	>20	8/10/2002
91	P129	*Termitomyces* sp.	NR4	>10	8/10/2002
92	ML001	*Stereum ostrea* (Blume & Nees) Fr.	NR3	>10	5/6/2003
93	ML002	*Stereum ostrea* (Blume & Nees) Fr.	NR3	>10	5/6/2003
94	ML003	*Stereum ostrea* (Blume & Nees) Fr.	NR3	>10	5/6/2003
95	ML004	*Polyporus* sp.	NR3	5	5/6/2003
96	ML005	*Stereum* sp.	NR3	8	5/6/2003
97	ML006	*Microporus* sp.	NR3	>10	5/6/2003
98	ML007	*Polyporus* sp.	NR3	>15	5/6/2003
99	ML008	*Hymenochaete* sp.	NR3	>10	5/6/2003
100	ML009	*Psathyrella* sp.	NR3	>10	5/6/2003
101	ML011	*Russula* cf. *heterophylla* (Fr.) Fr.	NR3	8	13/7/2003
102	ML012	*Russula aeruginea* Lindbl.	NR3	2	13/7/2003
103	ML013	*Polyporus* sp.	NR3	2-5	13/7/2003
104	ML014	*Amanita hemibapha* subsp. *javanica* Corner & Bas	NR3	1	13/7/2003
105	ML015	*Cantharellus minor* Peck.	NR3	>200	13/7/2003
106	ML016	*Cantharellus* cf. *cibarius* Fr.	NR3	>50	13/7/2003
107	ML018	*Xerocomus* sp.	NR3	2	13/7/2003
108	ML019	*Russula* sp.	NR3	3	13/7/2003
109	ML020	*Amanita* sect. *Vaginatae*	NR3	2	13/7/2003
110	ML023	*Amanita* sect. *Vaginatae*	NR3	3	13/7/2003

Table 11. (Continued)

No.	Code no.	Fungal species	Location[1]	No. of specimen	Collection date
111	ML024	*Amanita* sp.	NR3	2	13/7/2003
112	ML025	*Amanita* cf. *batterae* Boud.	NR3	1	13/7/2003
113	ML026	*Boletus* sp.	NR3	3	12/7/2003
114	ML028	*Xerocomus* sp.	NR3	2	12/7/2003
115	ML029	*Tylopilus* sp.	NR3	4	12/7/2003
116	ML030	*Boletus* sp.	NR3	2	12/7/2003
117	ML031	*Boletus* sp.	NR3	1	12/7/2003
118	ML032	*Gyroporus* sp.	NR3	1	12/7/2003
119	ML033	*Gyroporus* sp.	NR3	1	12/7/2003
120	ML034	*Strobilomyces mollis* Corner gp.	NR3	1	12/7/2003
121	ML037	*Russula* sp.	NR3	2	12/7/2003
122	ML038	*Lactarius* sp.	NR3	2	12/7/2003
123	ML039	*Russula luteotacta* Rea.	NR3	3	22/8/2003
124	ML040	*Russula alboareolata* Hongo.	NR3	3	12/7/2003
125	ML041	*Lactarius volemus* Fr.: Fr.	NR3	4	12/7/2003
126	ML042	*Cantharellus cibarius* Fr.	NR3	10-15	12/7/2003
127	ML043	*Russula* sp.	NR3	3	12/7/2003
128	ML044	*Russula delica* Fr.	NR3	5	12/7/2003
129	ML046	*Cantharellus* sp.	NR2	10-20	24/7/2003
130	ML047	*Russula* cf. *heterophylla* (Fr.) Fr.	NR2	3	24/7/2003
131	ML048	*Russula aeruginea* Lindbl. Sing.	NR1	3	24/7/2003
132	ML049	*Russula ochroleuca* Pers.	NR1	2-3	24/7/2003
133	ML050	*Russula delica* Fr.	NR1	2	24/7/2003
134	ML051	*Russula alboareolata* Hongo.	NR2	3-5	24/7/2003
135	ML052	*Russula* sp.	NR1	20-25	26/7/2003
136	ML053	*Russula* cf. *heterophylla* (Fr.) Fr.	NR1	2	26/7/2003
137	ML054	*Russula* sp.	NR1	2	26/7/2003
138	ML055	*Lentinus* sp.	NR1	4	26/7/2003

Table 11. (Continued)

No.	Code no.	Fungal species	Location[1]	No. of specimen	Collection date
139	ML056	*Termitomyces* sp.	NR1	10-15	26/7/2003
140	ML057	*Termitomyces microcarpus* (Berk. et Br.) Heim	BL1	>1000	26/7/2003
141	ML058	*Mycoamaranthus* sp.	BL1	3	26/7/2003
142	ML059	*Mycoamaranthus* sp.	BL1	>10	26/7/2003
143	ML060	*Coprinus* sp.	NR1	30-40	26/7/2003
144	ML061	*Chamaemyces fracidus* (Fr.) Donk	NR1	3	26/7/2003
145	ML062	*Lycoperdon* sp.	NR1	>20	26/7/2003
146	ML063	*Lycoperdon* sp.	NR1	10-15	26/7/2003
147	ML064	*Scleroderma* sp.	NR1	3-5	26/7/2003
148	ML066	*Termitomyces* sp.	NR2	3-5	4/8/2003
149	ML069	*Cantharellus minor* Peck.	NR2	>500	4/8/2003
150	ML070	*Cantharellus minor* Peck.	NR2	500-1000	4/8/2003
151	ML071	*Marasmius* sp.	NR2	5-10	4/8/2003
152	ML075	*Macrolepiota* sp.	NR1	>10	6/8/2003
153	ML078	*Schizophyllum commune* Fr.: Fr.	NP	>80	18/8/2003
154	ML083	*Termitomyces clypeatus* R. Heim	NR1	5-10	18/8/2003
155	ML084	*Termitomyces clypeatus* R. Heim	NR1	5	18/8/2003
156	ML085	*Termitomyces aurantiacus* R. Heim	NR1	4	18/8/2003
157	ML086	*Termitomyces aurantiacus* R. Heim	NR1	5-10	18/8/2003
158	ML087	*Termitomyces aurantiacus* R. Heim	NR1	5	18/8/2003
159	ML088	*Termitomyces* sp.	NR1	3	18/8/2003
160	ML089	*Termitomyces* sp.	NR1	3	18/8/2003
161	ML090	*Stereopsis radicans* (Berk.) Reid	NR2	10-20	21/8/2003
162	ML093	*Scleroderma* sp.	NR3	5	19/8/2003
163	ML094	*Leucocoprinus* sp.	NR3	5-10	19/8/2003
164	ML095	*Lentinellus* sp.	NR2	20-30	21/8/2003
165	ML096	*Russula alboareolata* Hongo.	NR2	3	21/8/2003
166	ML097	*Russula aeruginea* Lindbl.	NR3	3	21/8/2003

Table 11. (Continued)

No.	Code no.	Fungal species	Location[1]	No. of specimen	Collection date
167	ML098	*Lycoperdon* sp.	NR3	2	22/8/2003
168	ML099	*Scleroderma sinnamariense* Mont.	NR3	3	22/8/2003
169	ML100	*Russula* sp.	NR3	2	22/8/2003
170	ML101	*Tylopilus plumbeoviolaceus* (Snell & Dick) Sing.	NR3	3	22/8/2003
171	ML102	*Lepista sordida* (Fr.) Sing.	NR3	>10	22/8/2003
172	ML103	*Boletus* sp.	NR3	2	22/8/2003
173	ML104	*Trogia adelphus* Corner	NR2	20-40	21/8/2003
174	ML105	*Lepiota* sp.	NR3	>10	22/8/2003
175	ML106	*Macrolepiota* sp.	NR3	5	22/8/2003
176	ML107	*Amanita* sect. *Vaginatae*	NR3	5	22/8/2003
177	ML108	*Laccaria* sp.	NR3	>40	22/8/2003
178	ML109	*Heimannomyces splendidissima* Watl.	NR2	2	21/8/2003
179	ML110	*Amanita* sect. *Vaginatae*	NR3	1	21/8/2003
180	ML111	*Amanita* cf. *nauseosa* (Wakef.) Reid	NR3	2	22/8/2003
181	ML112	*Geastrum triptex* Jungh.	NR3	2	22/8/2003
182	ML113	*Amanita* sect. *Vaginatae*	NR3	3	22/8/2003
183	ML114	*Macrolepiota* sp.	NR1	5	22/8/2003
184	ML115	*Lepiota* sp.	NR1	7	25-27/8/2003
185	ML116	*Leucocoprinus* sp.	NR1	5	25-27/8/2003
186	ML117	*Leucocoprinus* sp.	NR1	5	25-27/8/2003
187	ML118	*Amanita* sect. *Vaginatae*	NR3	1	22/8/2003
188	ML120	*Leucocoprinus* sp.	NR1	5-10	25-27/8/2003
189	ML125	*Entoloma* sp.	NR1	4-5	25-27/8/2003
190	ML126	*Leucocoprinus* sp.	NR1	5-10	25-27/8/2003
191	ML127	*Lepiota* sp.	NR1	>5	25-27/8/2003
192	ML128	*Leucoagaricus* sp.	NR1	>10	25-27/8/2003
193	ML129	*Lepiota* sp.	NR1	>5	25-27/8/2003
194	ML130	*Lepiota* sp.	BL2	>5	24/8/2003

Table 11. (Continued)

No.	Code no.	Fungal species	Location[1]	No. of specimen	Collection date
195	ML131	*Termitomyces* sp.	NR1	10-20	25-27/8/2003
196	ML132	*Cantharellus* sp.	NR3	5-10	19/8/2003
197	ML133	*Russula* sect. *plarantae*, prob. *pseudodelica*	NR3	>10	19/8/2003
198	ML134	*Cantharellus minor* Peck.	NR3	>200	19/8/2003
199	ML135	*Hiemiella* prob. *Retispora* (Pat. & Baker) Boedijn	BL3	7-10	24/8/2003
200	ML136	*Pycnoporus cinnabarius* (Jacq.: Fr.) P. Karst.	NR4	>5	24/8/2003
201	ML137	*Amanita* sp.	BL	>50	24/8/2003
202	ML138	*Chlorophyllum molybdites* (Meyer: Fr.) Massee	NR1	>15	24/8/2003
203	ML139	*Leucoagaricus* sp.	NR1	>10	24/8/2003
204	ML140	*Russula* sect. *Foetinae*	BL3	>10	24/8/2003
205	ML142	*Russula luteotacta* Rea.	NR1	5	22/8/2003
206	ML143	*Cantharellus minor* Peck.	BL3	>20	24/8/2003
207	ML144	*Termitomyces aurantiacus* R. Heim	NR1	>10	24/8/2003
208	ML145	*Termitomyces* sp.	NR1	5-10	24/8/2003
209	ML146	*Auricularia polytricha* (Mont.) Sacc.	BL2	5-10	24/8/2003
210	ML147	*Russula* sect. *Plorantae brevipes*/*delica*	BL2	>40	24/8/2003
211	ML148	*Russula* sp.	BL3	5-10	24/8/2003
212	ML149	*Russula anthracina* Romagn.	BL3	5	24/8/2003
213	ML150	*Schizophyllum commune* Fr.: Fr.	NR2	>15	24/8/2003
214	ML151	Agaricoid	NR2	3-5	21/8/2003
215	ML152	*Clavulina cristata* var. *cineroides* or *cinerea*	BL3	>40	24/8/2003
216	ML153	*Podoscypha nitidula* (Berk.) Pat. in Duss	BL3	>10	24/8/2003
217	ML154	*Macrolepiota gracilenta* (Krombh.) Sing.	NP	5	23/8/2003
218	ML159	*Clavulina cristata* (Fr.) Schroet.	NR1	>30	25-27/8/2003

Code: P= Mushroom collection in year 2002, ML= Mushroom collection in year 2003

[1]**Location:** NR= Nakhon Ratchasima Province: NR1= Natural forests in suburb area, NR2= Nong-raweing Plant Genetics Forest, Muang District, NR3= Pak Thong Chai District, NR4=Wang Num Keaw District, and NR5= Local markets, Muang District, BL= Burirum Province: BL1= Nong Ki District, BL2= Nang Rong District, BL3= Chalermprakiat District, and BL4= Lam Plai Mat District, NP= Nakhon Pathom, KB= Kanchanaburi.

APPENDIX E

DETERMINATION OF LECTINS ACCUMULATED IN MUSHROOM SPECIMENS

Table 12. Hemagglutination activity against human and animal red blood cellls of crude extracts of 218 mushroom specimens.

Mushroom Species	Source[1]	Code no.	Activity against red blood cells (Titer)						
			Human			Animal			
			A	B	O	Sheep	Rat	Goose	Rabbit
Agaricaceae									
Agaricoid	NR2	ML151	8	8	8H	4	256PH	16	16
Chamaemyces fracidus (Fr.) Donk.	NR1	ML061	4	1.5	ND	6	24	1	12
Chlorophyllum molybdites (Meyer: Fr.) Massee	NR1	ML138	H	H	1024H	H	72PH	H	160
Heimannomyces splendidissima Watl.	NR2	ML109	H	H	H	H	48H	H	6
Leucocoprinus sp. (1)	NR1	P022	ND	ND	ND	ND	ND	ND	ND
Leucocoprinus sp. (2)	NR1	P029	8	4	8	32	64	64	1
Leucocoprinus sp. (3)	NR1	P034	16	16	64	32	64	32	1
Leucocoprinus sp. (4)	NR1	P094	32	64	64	8	64	64	1
Leucocoprinus sp. (5)	NR3	ML094	ND	ND	ND	ND	4H	ND	4H
Leucocoprinus sp. (6)	NR1	ML116	1.5H	H	20H	ND	128H	ND	20
Leucocoprinus sp. (7)	NR1	ML117	1024H	ND	ND	ND	PH	ND	32
Leucocoprinus sp. (8)	NR1	ML120	1024H	ND	ND	ND	ND	ND	ND
Leucocoprinus sp. (9)	NR1	ML126	128	256	192	64	PH	1024	256
Leucoagaricus sp. (10)	NR1	ML128	1024H	H	H	48	320H	H	96
Leucoagaricus sp. (11)	NR1	ML139	64	64	96	16	192H	128	192
Lepiota sp. (1)	NR1	P023	ND	ND	ND	ND	16	ND	ND
Lepiota sp. (2)	NR1	P026	ND	ND	ND	ND	32	ND	ND
Lepiota sp. (3)	NR1	P030	ND	ND	ND	ND	64	ND	1
Lepiota sp. (4)	NR1	P037	H	H	H	PH	96	ND	H
Lepiota sp. (5)	NR3	ML105	ND	ND	ND	ND	16	ND	18
Lepiota sp. (6)	NR1	ML115	ND	ND	ND	H	192PH	ND	24
Lepiota sp. (7)	NR1	ML127	64H	130	33H	288H	640H	32	32H
Lepiota sp. (8)	NR1	ML129	12H	576H	ND	2H	96H	2H	64
Lepiota sp. (9)	BL2	ML130	H	4H	3H	ND	288H	ND	32H
Leucocoprinus cf. *birnbaumii* (Corda) Sing.	NR1	P093	ND	ND	ND	ND	ND	ND	ND
Macrolepiota dolichaula (Berk. & Br.) Pegler & Ray	NR1	P003	ND	ND	ND	ND	2	ND	ND
Macrolepiota gracilenta (Krombh.) Sing.	BL3	ML154	36H	H	ND	ND	ND	ND	ND
Macrolepiota sp.(1)	NR1	P095	8	16	16	2	32	512	1

Table 12. (Continued)

Mushroom species	Source[1]	Code no.	Activity against red blood cells (Titer)						
			Human			Animal			
			A	B	O	Sheep	Rat	Goose	Rabbit
Agaricaceae									
Macrolepiota sp. (2)	NR1	P113	**512**	256	256	64	1024	32	PH
Macrolepiota sp. (3)	NR1	ML075	ND	ND	ND	H	8	ND	ND
Macrolepiota sp. (4)	NR3	ML106	4	12	8	10	48	16	32
Macrolepiota sp. (5)	NR1	ML114	4H	2H	3H	4H	128	4H	4H
Amanitaceae									
Amanita cf. *batterae* Boud.	NR3	ML025	ND	ND	ND	ND	ND	ND	ND
Amanita cf. *nauseosa* (Wakef.) Reid	NR3	ML111	96	192	1024	32	1024	8H	1024
Amanita hemibapha subsp. *javanica* Corner & Bas (1)	NR5	ML014	ND	ND	ND	H	ND	ND	1
Amanita sect. *Vaginatae* (1)	NR3	ML020	4H	8H	16H	ND	96H	ND	128H
Amanita sect. *Vaginatae* (2)	NR3	ML023	ND	ND	ND	2.5H	H	ND	H
Amanita sect. *Vaginatae* (3)	NR3	ML107	ND	ND	ND	ND	ND	ND	ND
Amanita sect. *Vaginatae* (4)	NR3	ML110	2H	2H	2H	4H	128H	2H	6H
Amanita sect. *Vaginatae* (5)	NR3	ML113	1.5H	H	2H	3H	48H	2H	4H
Amanita sect. *Vaginatae* (6)	NR3	ML118	128H	ND	ND	16	256PH	ND	160
Amanita sp. (1)	NR5	P042	H	ND	ND	ND	4	ND	PH
Amanita sp. (2)	BL1	P054	H	H	H	ND	H	H	H
Amanita sp. (3)	BL1	P069	H	H	PH	ND	6	H	H
Amanita sp. (4)	BL1	P077	ND	ND	ND	ND	2	H	PH
Amanita sp. (5)	NR1	P097	8	16	8	4	96	8	1
Amanita sp. (6)	NR3	ML024	ND	ND	ND	ND	ND	ND	ND
Amanita sp. (7)	BL2	ML137	ND	ND	ND	H	32H	ND	6H
Amanita sp. (8)	NR5	P043	H	ND	ND	ND	8	ND	PH
Auriculariaceae									
Auricularia polytricha (Mont.) Sacc. (1)	NR1	P001	ND	ND	ND	ND	2	ND	ND
Auricularia polytricha (Mont.) Sacc. (2)	NP	P108	ND	ND	ND	ND	8	PH	ND
Auricularia polytricha (Mont.) Sacc. (3)	NR1	P016	ND	ND	ND	H	2	ND	ND
Auricularia polytricha (Mont.) Sacc. (4)	BL2	ML146	H	1024H	48H	ND	ND	ND	ND
Auricularia sp.	NP	P111	ND	ND	ND	ND	2	ND	ND

Table 12. (Continued)

Mushroom species	Source[1]	Code no.	Activity against red blood cells (Titers)						
			Human				Animal		
			A	B	O	Sheep	Rat	Goose	Rabbit
Bolbitiaceae									
Agrocybe cylindraceae (DC.: Fr.) Maire (1)	NR1	P007	H	PH	H	H	64	64	1
Agrocybe cylindraceae (DC.: Fr.) Maire (2)	NR1	P087	ND	ND	ND	ND	2	ND	ND
Boletaceae									
Boletus sp. (1)	NR3	ML026	4	8	8	4	384	64	48
Boletus sp. (2)	NR3	ML030	24	32	32	48	576	128	512
Boletus sp. (3)	NR3	ML031	24	32	16	16	48	192	48
Boletus sp. (4)	NR3	ML103	24	64	64	48	128	768	768
Gyroporus sp. (1)	NR3	ML032	6H	2H	H	H	256	H	H
Gyroporus sp. (2)	NR3	ML033	ND	ND	ND	ND	ND	ND	512
Hiemiella prob. *retispora* (Pat. & Baker) Boedijn	BL3	ML135	ND	ND	2H	ND	ND	1024	288H
Strobilomyces mollis Corner gp.	NR3	ML034	ND	ND	ND	8.5	512	ND	32
Tylopilus plumbeoviolaceus (Snell & Dick) Sing.	NR3	ML101	ND	ND	ND	ND	ND	ND	2.5H
Tylopilus sp. (1)	NR1	P049	ND	ND	ND	ND	ND	ND	PH
Tylopilus sp. (2)	NR1	ML029	ND	4	2	ND	ND	ND	ND
Xerocomus sp.(1)	NR3	ML018	ND	ND	ND	H	132	ND	5
Xerocomus sp.(2)	NR3	ML028	16	24	32	32	64	256	68
Cantharellaceae									
Craterellus sp.	NR1	P098	H	PH	H	PH	12	PH	ND
Cantharellus cf. *cibarius* Fr. (1)	NR1	ML016	ND	ND	ND	ND	640	ND	288
Cantharellus cf. *cibarius* Fr. (2)	NR3	ML042	ND	ND	ND	H	0.5	1	16
Cantharellus minor Peck. (1)	NR1	P041	ND	ND	ND	ND	16	ND	PH
Cantharellus minor Peck. (2)	BL1	P078	ND	ND	ND	ND	8	PH	1
Cantharellus minor Peck. (3)	NR3	ML015	ND	ND	1	H	8	1.5	272
Cantharellus minor Peck. (4)	NR2	ML069	ND	ND	ND	H	1	1.5H	H
Cantharellus minor Peck. (5)	NR2	ML070	ND	ND	ND	ND	1.5	ND	H
Cantharellus minor Peck. (6)	NR3	ML134	ND	H	H	ND	8PH	ND	2
Cantharellus minor Peck. (7)	BL3	ML143	ND	ND	ND	ND	96PH	ND	16
Cantharellus sp. (1)	NR2	ML046	ND	ND	ND	24H	288	1	544

Table 12. (Continued)

Mushroom species	Source[1]	Code no.	Activity against red blood cells (Titer)						
			Human			Animal			
			A	B	O	Sheep	Rat	Goose	Rabbit
Cantharellaceae									
Cantharellus sp. (2)	NR3	ML132	8H	10H	40H	H	1024PH	2	32H
Cariolaceae									
Pycnoporus sp.	NR5	P090	16	64	64	PH	H	H	H
Pycnoporus cinnabarius (Jacq.: Fr.) P. Karst.	NR3	ML136	ND	H	H	8H	64	H	2H
Clavariaceae									
Clavulina cristata var. *cineroides* or *cinerea*	NR2	ML152	1.5H	2H	3H	4	64H	ND	16
Clavulina cristata (Fr.) Schroet.	NR1	ML159	8	4	4	ND	ND	ND	ND
Coprinaceae									
Coprinus sp.	NR1	ML060	4	2	4	H	64	ND	ND
Psathyrella sp.	NR3	ML009	2	2	2	4	48	32	128
Entolomataceae									
Entoloma sp.	NR1	ML125	1024H	1024H	1024H	8	1024H	ND	10
Geastraceae									
Geastrum triptex Jungh.	NR3	ML112	ND	ND	ND	ND	48	ND	2
Helvellaceae									
Stereopsis radicans (Berk.) Raid	NR2	ML090	ND	ND	ND	ND	10	ND	24H
Hymanochaetaceae									
Hymenochaete sp.	NR3	ML008	ND	ND	ND	2.5H	16	ND	516
Lycoperdaceae									
Mycoamaranthus sp. (1)	BL1	ML058	16H	16H	2H	12	256	2	256
Mycoamaranthus sp. (2)	BL1	ML059	ND	ND	ND	ND	ND	ND	ND
Lycoperdon sp. (1)	NR1	P096	ND	ND	ND	ND	256	ND	ND
Lycoperdon sp. (2)	NR1	ML062	ND	ND	2H	H	544	ND	2
Lycoperdon sp. (3)	NR1	ML063	ND	ND	ND	H	ND	ND	6
Lycoperdon sp. (4)	NR3	ML098	ND	ND	ND	ND	12	ND	ND

Table 12. (Continued)

Mushroom species	Source[1]	Code no.	Activity against red blood cells (Titer)						
			Human			Animal			
			A	B	O	Sheep	Rat	Goose	Rabbit
Peniophoraceae									
Stereum ostrea (Blume & Nees) Fr. (1)	NR3	ML001	ND	ND	ND	ND	4	ND	16
Stereum ostrea (Blume & Nees) Fr. (2)	NR3	ML002	ND	ND	ND	ND	16	ND	6
Stereum ostrea (Blume & Nees) Fr. (3)	NR3	ML003	ND	ND	ND	H	64	ND	96
Stereum sp.	NR3	ML005	ND	ND	ND	H	32	ND	6
Pleurotaceae									
Lentinellus sp.	NR2	ML095	3H	1	2	1.5H	24	H	8
Lentinus sp .(1)	NR5	P089	PH	ND	PH	2	64	PH	1
Lentinus sp .(2)	NP	P109	1.5	ND	ND	H	32	ND	1
Lentinus sp. (3)	NR1	ML055	3H	2H	H	H	1024	ND	3
Lentinula edodes (Berk.) Pegler (1)	NR1	P006	ND	ND	ND	ND	ND	ND	ND
Lentinula edodes (Berk.) Pegler (2)	NP	P106	ND	ND	ND	ND	ND	ND	ND
Lentinus polychrous Lev.	NR1	P018	PH	PH	PH	H	8	H	ND
Lentinula polychrous Lev.	NP	P107	PH	ND	1	ND	H	ND	1
Lentinula polychrous Lev.	NR5	P114	ND	ND	ND	ND	16	PH	ND
Lentinula squarrosulus Mont. (1)	NR5	P012	16	32	32	4	H	H	H
Lentinula squarrosulus Mont. (2)	NR1	P002	2	4	2	2	H	8	1
Lentinula squarrosulus Mont. (3)	NR1	P028	PH	PH	PH	ND	H	PH	H
Lentinula sp.	NR5	P014	ND	ND	ND	ND	16	ND	H
Pleurotus citrinopileatus Sing.	NP	P103	ND	ND	ND	ND	PH	PH	ND
Pleurotus cornucopiae (Paul: Pers) Rolland (1)	NR1	P017	ND	ND	ND	H	8	ND	H
Pleurotus cornucopiae (Paul: Pers) Rolland (2)	NR1	P035	ND	4	4	ND	32	ND	ND
Pleurotus cystidiosus O.K. Miller (1)	NR1	P009	ND	ND	ND	ND	ND	ND	ND
Pleurotus djamor (Fr.) Boedijn (1)	NR1	P036	1	2	2	ND	8	ND	ND
Pleurotus djamor (Fr.) Boedijn (2)	NP	P102	ND	ND	ND	H	PH	PH	ND
Pleurotus ostreatus (Jacq.: Fr.) P. Kumm.	NP	P110	ND	ND	ND	ND	1	PH	ND
Pleurotus sajor-caju (Fr.) Sing. (1)	NR5	P015	H	H	H	H	PH	PH	H
Pleurotus sajor-caju (Fr.) Sing. (2)	NR5	P092	H	H	H	H	H	ND	H
Pleurotus sajor-caju (Fr.) Sing. (3)	NR5	P122	H	H	H	H	PH	PH	H

Table 12. (Continued)

Mushroom species	Source[1]	Code no.	Activity against red blood cells (Titer)						
			Human			Animal			
			A	B	O	Sheep	Rat	Goose	Rabbit
Pleurotaceae									
Pleurotus sp. (Butan) (1)	NR1	P008	H	H	H	H	ND	ND	ND
Pleurotus sp. (Butan) (2)	NP	P104	H	H	H	H	ND	H	H
Pleurotus sp. (Hungarian) (1)	NR1	P004	H	H	H	H	H	H	H
Pleurotus sp. (Hungarian) (2)	NR5	P112	H	H	H	H	H	ND	H
Podoscyphaceae									
Podoscypha nitidula (Berk.) Pat. in Duss	BL3	ML153	ND	ND	ND	ND	16	ND	6
Polyporaceae									
Microporus sp.	NR3	ML006	ND	ND	ND	H	16	ND	128
Polyporus sp. (1)	NR3	ML004	ND	ND	ND	ND	8	ND	4
Polyporus sp. (2)	NR3	ML007	ND	ND	ND	H	ND	ND	12
Polyporus sp. (3)	NR3	ML013	ND	ND	ND	ND	96	ND	256
Pluteaceae									
Volvariella volvacea (Bull.: Fr.) Sing.	NR5	P013	ND	ND	ND	H	64	ND	H
Russulaceae									
Lactarius volemus Fr.: Fr.	NR3	ML041	32	256	128	32	32	512	256
Lactarius sp. (1)	NR1	P032	6	2	8	ND	64	4	1
Lactarius sp. (2)	NR3	ML038	1.5	1.5	2.5H	H	16	H	4
Russula aeruginea Lindbl. (1)	NR3	ML012	ND	ND	ND	ND	ND	ND	ND
Russula aeruginea Lindbl. (2)	NR1	ML048	ND	ND	ND	ND	ND	ND	384
Russula aeruginea Lindbl. (3)	NR3	ML097	8	16	10	4	32	16	24
Russula anthracina Romagn.	BL3	ML149	3H	1024H	H	ND	272	ND	12
Russula alboareolata Hongo. (1)	BL1	P072	ND	ND	ND	H	ND	ND	H
Russula alboareolata Hongo. (2)	NR3	ML040	ND	ND	ND	ND	ND	ND	1
Russula alboareolata Hongo. (3)	NR2	ML051	ND	ND	ND	ND	160	1	12
Russula alboareolata Hongo. (4)	NR2	ML096	3H	8	8	16H	8H	8H	16H
Russula delica Fr. (1)	NR3	ML044	ND	ND	ND	H	10	4	16
Russula delica Fr. (2)	NR1	ML050	ND	ND	ND	H	ND	ND	8
Russula cf. *cyanoxantha* (Schaeff: Schw.) Fr.	BL1	P066	ND	ND	ND	ND	ND	ND	ND

Table 12. (Continued)

Mushroom species	Source[1]	Code no.	Activity against red blood cells (Titer)						
			Human			Animal			
			A	B	O	Sheep	Rat	Goose	Rabbit
Russulaceae									
Russula cf. *heterophylla* (Fr.) Fr. (1)	NR3	ML011	ND	ND	ND	ND	260	ND	20
Russula cf. *heterophylla* (Fr.) Fr. (2)	NR2	ML047	ND	ND	ND	H	ND	ND	4
Russula cf. *heterophylla* (Fr.) Fr. (3)	NR1	ML053	4H	3H	ND	H	ND	3	8
Russula luteotacta Rea. (1)	NR3	ML039	ND	3.5	3.5	2	2	2	4
Russula luteotacta Rea. (2)	NR3	ML100	8	8	6	4	32	32	16
Russula luteotacta Rea. (3)	NR2	ML142	8	8	16	H	640H	64	48
Russula ochroleuca Pers.	NR1	ML049	ND	ND	ND	ND	ND	ND	10
Russula sect. *Foetinae*	BL3	ML140	8H	16	24H	2	64H	2	3
Russula sect. *Nigricantae* Fr.	BL1	P071	ND	ND	ND	ND	ND	ND	H
Russula sect. *Plorantae brevipes/delica*	BL2	ML147	ND	9H	ND	ND	ND	ND	ND
Russula sect. *Plorantiae* prob. *pseudodelica*	NR3	ML133	8H	32H	64H	8	80	ND	48
Russula sp. (1)	BL1	P070	PH	PH	PH	PH	ND	PH	H
Russula sp. (2)	BL1	P074	8	H	H	H	5	H	H
Russula sp. (3)	BL1	P075	8	16	32	2	16	16	1
Russula sp. (4)	BL1	P076	ND	ND	ND	H	2	ND	ND
Russula sp. (5)	NR5	P118	ND	ND	ND	H	2	ND	ND
Russula sp. (6)	NR5	P119	ND	ND	ND	ND	ND	ND	ND
Russula sp. (7)	BL4	P120	ND	ND	ND	ND	ND	ND	ND
Russula sp. (8)	NR3	ML019	1	2	2	H	1.5	ND	1024
Russula sp. (9)	NR3	ML037	ND	ND	ND	H	128	ND	1024
Russula sp. (10)	NR3	ML043	32	48	24	64	128	512	384
Russula sp. (11)	NR1	ML052	ND	ND	ND	H	ND	ND	24
Russula sp. (12)	NR1	ML054	4	12	4	2	32H	16	16
Russula sp. (13)	BL3	ML148	ND	ND	ND	ND	ND	ND	1
Schizophyllaceae									
Schizophyllum commune Fr.: Fr. (1)	NP	P105	PH	16	8	ND	H	ND	H
Schizophyllum commune Fr.: Fr. (2)	NP	ML078	16	32	12	ND	H	ND	16
Schizophyllum commune Fr.: Fr. (3)	NR2	ML150	32	32	8	ND	H	ND	H

Table 12. (Continued)

Mushroom species	Source[1]	Code no.	Activity against red blood cells (Titer)						
			Human			Animal			
			A	B	O	Sheep	Rat	Goose	Rabbit
Sclerodermataceae									
Scleroderma sinnamariense Mont.	NR3	ML099	ND	ND	ND	ND	ND	ND	ND
Scleroderma sp. (1)	NR1	ML064	ND	ND	ND	256H	2	ND	H
Scleroderma sp. (2)	NR3	ML093	1024H	ND	ND	ND	12H	ND	ND
Tricholomataceae									
Flammulina velutipes (Fr.) P. Karst (1)	NR1	P010	PH	ND	ND	ND	H	PH	H
Flammulina velutipes (Fr.) P. Karst (2)	NR5	P121	H	H	H	H	H	H	H
Flammulina velutipes (Fr.) P. Karst (3)	NR1	P126	H	H	H	H	H	32	H
Flammulina velutipes (Fr.) P. Karst (4)	NR1	P127	H	H	H	1	H	32	H
Laccaria sp. (1)	NR1	P025	ND	8	16	ND	32	ND	ND
Laccaria sp. (2)	KB	P100	PH	12	16	ND	H	ND	H
Laccaria sp. (3)	NR5	P115	16	8	8	H	H	PH	1
Laccaria sp. (4)	BL4	P123	H	H	H	H	H	PH	1
Laccaria sp. (5)	NR3	ML108	1	4	6	2	16	1	128
Lepista sordida (Fr.) Sing.	NR3	ML102	H	ND	ND	6	32	48	128
Macrocybe sp.	NR1	P011	PH	PH	PH	PH	128	PH	H
Macrocybe crassum	NR5	P019	ND	ND	ND	H	48	ND	1
Marasmius sp. (1)	NR1	P099	H	H	H	H	H	H	H
Marasmius sp. (2)	NR2	ML071	ND	ND	2H	H	ND	ND	512
Termitomyces sp. (1)	NR1	P005	ND	ND	ND	ND	64	PH	H
Termitomyces sp. (2)	NR1	P020	ND	ND	ND	ND	ND	ND	H
Termitomyces sp. (3)	BL1	P065	ND	ND	PH	ND	ND	ND	H
Termitomyces sp. (4)	BL1	P067	ND	ND	PH	ND	32	ND	ND
Termitomyces sp. (5)	BL1	P068	ND	ND	ND	ND	96	ND	ND
Termitomyces sp. (6)	KB	P101	ND	ND	ND	ND	64	PH	ND
Termitomyces sp. (7)	NR5	P116	ND	ND	ND	H	PH	PH	PH
Termitomyces sp. (8)	NR5	P117	ND	ND	ND	PH	PH	PH	PH
Termitomyces sp. (9)	NR1	P124	PH	PH	1.5	PH	H	PH	H

Table 12. (Continued)

Mushroom species	Source[1]	Code no.	Activity against red blood cells (Titer)						
			Human			Animal			
			A	B	O	Sheep	Rat	Goose	Rabbit
Tricholomataceae									
Termitomyces sp. (10)	NR1	P125	ND	PH	ND	ND	1	PH	H
Termitomyces sp. (11)	NR4	P128	PH	PH	1	H	160	PH	1
Termitomyces sp. (12)	NR4	P129	1	1	1.5	H	H	PH	ND
Termitomyces sp. (13)	NR1	ML056	ND	ND	ND	H	1	ND	2H
Termitomyces sp. (14)	NR2	ML066	ND	ND	ND	H	3	ND	1.5H
Termitomyces sp. (15)	NR1	ML088	1.5H	ND	ND	H	90	2H	2H
Termitomyces sp. (16)	NR1	ML089	H	H	H	H	48	2H	2H
Termitomyces sp. (17)	NR1	ML131	H	ND	4H	H	4	ND	64
Termitomyces sp. (18)	NR1	ML145	ND	ND	ND	ND	ND	ND	64H
Termitomyces aurantiacus R. Heim (1)	NR2	ML085	ND	ND	ND	H	H	H	H
Termitomyces aurantiacus R. Heim (2)	NR1	ML086	ND	H	H	1.5H	96	H	1.5H
Termitomyces aurantiacus R. Heim (3)	NR1	ML087	1.5H	H	H	H	8H	3H	64H
Termitomyces aurantiacus R. Heim (4)	NR1	ML144	ND	ND	H	ND	ND	ND	ND
Termitomyces clypeatus R. Heim (1)	NR1	ML083	1.5H	ND	ND	ND	ND	2H	2H
Termitomyces clypeatus R. Heim (2)	NR1	ML084	H	H	H	ND	ND	ND	H
Termitomyces microcarpus (Berk. et Br.) Heim	BL1	ML057	ND	ND	ND	ND	32	ND	ND
Trogia adelphus Corner	NR2	ML104	H	ND	H	ND	64	ND	32

H: Hemolysis, PH: Partial hemolysis, ND: Non-detected **1** Source: NR= Nakhon Ratchasima Province: NR1= Natural forests in suburb area, NR2= Nong-raweing Plant Genetics Forest, Muang District, NR3= Pak Thong Chai District, NR4=Wang Num Keaw District, and NR5= Local markets, Muang District, BL= Burirum Province: BL1= Nong Ki District, BL2= Nang Rong District, BL3= Chalermprakiat District, and BL4= Lam Plai Mat District, NP= Nakhon Pathom, KB= Kanchanaburi.

APPENDIX F

RESULTS FROM CYTOTOXICITY TEST

Table 13. Forty-five selected mushroom extracts from cytotoxic activity test against cancer cell lines by MTT colorimetric assay.

No.	Code.	Mushroom species	IC_{50} (µg/ml)	
			KB	**HeLa**
1	ML003	*Stereum ostrea* (Blume & Nees) Fr.	550	500
2	ML011	*Russula* cf. *heterophylla* (Fr.) Fr.	400	145
3	ML013	*Polyporus* sp.	200	200
4	ML015	*Cantharellus minor* Peck	68	68
5	ML016	*Cantharellus* cf. *cibarius* Fr.	3.55	3.551
6	ML020	*Amanita* sect. *Vaginatae*	2.5	0.14
7	ML026	*Boletus* sp.	120	600
8	ML028	*Xerocomus* sp.	180	450
9	ML030	*Boletus* sp.	420	400
10	ML034	*Strobilomyces mollis* Corner gp.	3	36.5
11	ML037	*Russula* sp.	125	125
12	ML039	*Russula luteotacta* Rea.	400	500
13	ML041	*Lactarius volemus* Fr.: Fr.	600	800
14	ML046	*Cantharellus* sp.	96	96
15	ML051	*Russula alboareolata* Hongo.	51.7	51.7
16	ML054	*Russula* sp.	21	21
17	ML055	*Lentinus* sp.	8	7
18	ML062	*Lycoperdon* sp.	483	400
19	ML071	*Marasmius* sp.	30	9.5
20	ML078	*Schizophyllum commune* Fr.: Fr.	20	350
21	ML086	*Termitomyces aurantiacus* R. Heim	97.3	97.3
22	ML088	*Termitomyces* sp.	1050	1980
23	ML089	*Termitomyces* sp.	6500	2500
24	ML095	*Lentinellus* sp.	170	650
25	ML097	*Russula aeruginea* Lindbl.	350	500
26	ML100	*Russula* sp.	1500	1100
27	ML102	*Lepista sordida* (Fr.) Sing.	76	76
28	ML103	*Boletus* sp.	480	320
29	ML104	*Trogia adelphus* Corner	120	320
30	ML106	*Macrolepiota* sp.	100	15
31	ML108	*Laccaria* sp.	900	300
32	ML111	*Amanita* cf. *nauseosa* (Wakef.) Reid	160	140
33	ML112	*Geastrum triptex* Jungh.	2000	1500
34	ML114	*Macrolepiota* sp.	1990	200
35	ML126	*Leucocoprinus* sp.	1500	2000
36	ML127	*Lepiota* sp.	380	200
37	ML128	*Leucoagaricus* sp.	55	1000
38	ML136	*Pycnoporus cinnabarius* (Jacq.: Fr.) P. Karst.	NT	220
39	ML138	*Chlorophyllum molybdites* (Meyer: Fr.) Massee	400	500
40	ML139	*Leucoagaricus* sp.	81	81
41	ML142	*Russula luteotacta* Rea.	40.3	40.3
42	ML149	*Russula anthracina* Romagn.	600	600
43	ML151	Agaricoid	140	350
44	ML152	*Clavulina cristata* var. *cineroides* or *cinerea*	280	800
45	ML159	*Clavulina cristata* (Fr.) Schroet.	2000	400

KB= Human epidermoid carcinoma, HeLa= Human cervical carcinoma

APPENDIX G

RESULTS OF SEQUENCE SIMILARITY

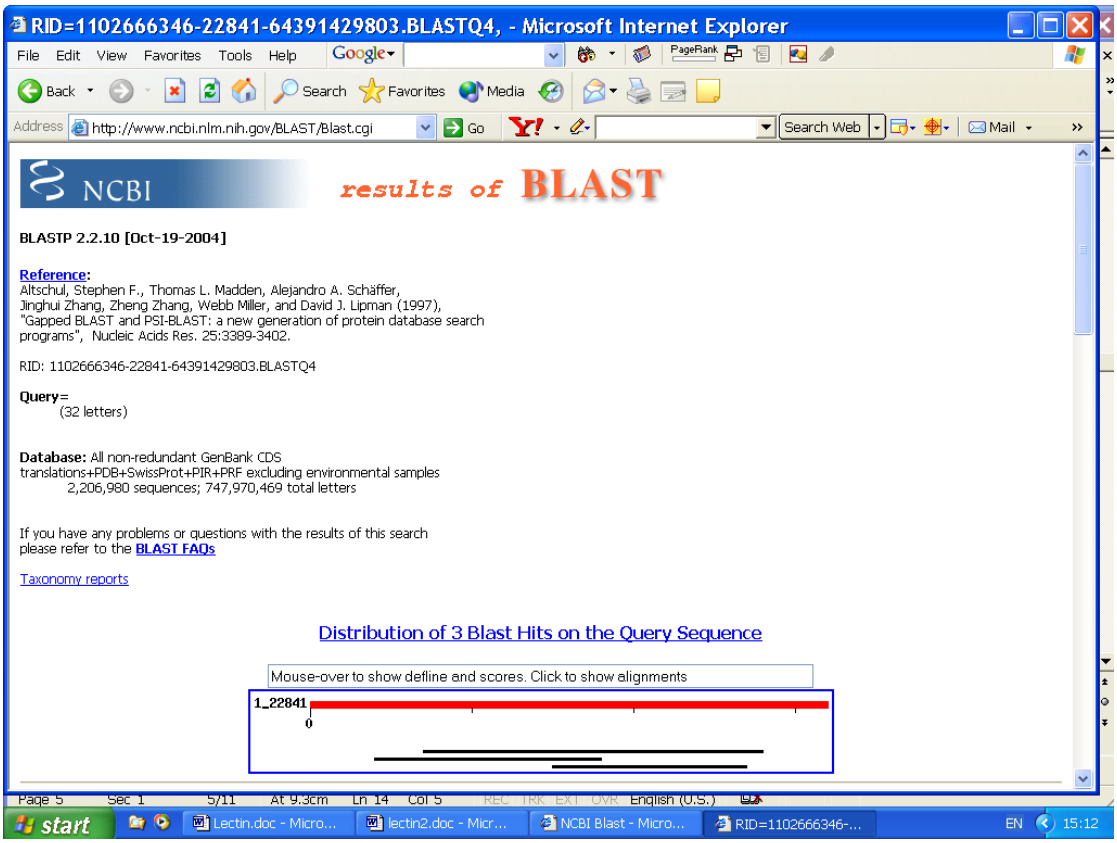


Figure 40. Results of sequence similarity of *Schizophyllum commune* lectin compared to other protein sequences from NCBI database (BLAST).

NCBI DATABASE

Sequence alignments of SCL
1-2-3 IQGCVGGRDE GTPIIGWDYXEX TFLADIKPGX
2-1-3 GTPIIGWDYXEX IQGCVGGRDE TFLADIKPGX
3-1-2 TFLADIKPGX IQGCVGGRDE GTPIIGWDYXEX
2-3-1 GTPIIGWDYXEX TFLADIKPGX IQGCVGGRDE
3-2-1 TFLADIKPGX GTPIIGWDYXEX IQGCVGGRDE
1-3-2 IQGCVGGRDE TFLADIKPGX GTPIIGWDYXEX

1-2-3 IQGCVGGRDE GTPIIGWDYXEX TFLADIKPGX
Lectin-all organisms

>gi|7671450|emb|CAB89390.1| lectin-like protein kinase-like [Arabidopsis thaliana]
gi|11346435|pir||T49986 lectin-like protein kinase-like - Arabidopsis thaliana
gi|15238190|ref|NP_196615.1| lectin protein kinase, putative [Arabidopsis thaliana]
Length = 651

Score = 24.3 bits (51), Expect = 1.7
Identities = 9/24 (37%), Positives = 13/24 (54%)

```
Query: 6   GGRDEGTPIIQWDYXEXTFLADIK 29
           GG  EG  ++ W++     L DIK
Sbjct: 237 GGVTEGNRLLSWEFSSSLELIDIK 260
```

>gi|18476512|gb|AAL47680.1| agglutinin [Marasmius oreades]
Length = 293

Score = 22.7 bits (47), Expect = 5.0
Identities = 7/18 (38%), Positives = 10/18 (55%)

```
Query: 6  GGRDEGTPIIQWDYXEXT 23
          G   +GTPI+ W +   T
Sbjct: 24 GSSSDGTPIVGWQFTPDT 41
```

gi|34915974|dbj|BAC87876.1| Ricin B-related lectin [Polyporus squamosus]
Length = 292

Score = 22.7 bits (47), Expect = 5.0
Identities = 8/19 (42%), Positives = 9/19 (47%)

```
Query: 6  GGRDEGTPIIQWDYXEXTF 24
          G    GTP+I WD     F
Sbjct: 27 GSGQNGTPVIAWDSNNDAF 45
```

Figure 40. (Continued)

2-1-3 GTPIIGWDYXEX IQGCVGGRDE TFLADIKPGX

>gi|18476512|gb|AAL47680.1| agglutinin [Marasmius oreades]
Length = 293

Score = 21.9 bits (45), Expect = 8.5
Identities = 6/9 (66%), Positives = 8/9 (88%)

```
Query: 1  GTPIIGWDY 9
          GTPI+GW +
Sbjct: 29 GTPIVGWQF 37
```

3-1-2 TFLADIKPGX IQGCVGGRDE GTPIIGWDYXEX

>gi|18476512|gb|AAL47680.1| agglutinin [Marasmius oreades]
Length = 293

Score = 25.0 bits (53), Expect = 1.00
Identities = 9/22 (40%), Positives = 12/22 (54%)

```
Query: 8  PGXIQGCVGGRDEGTPIIGWDY 29
          P  I    G   +GTPI+GW +
Sbjct: 16 PSAIDLKDGSSSDGTPIVGWQF 37
```

>gi|7263614|emb|CAB81580.1| putative protein [Arabidopsis thaliana]
Length = 1113

Score = 22.3 bits (46), Expect = 6.5
Identities = 7/15 (46%), Positives = 11/15 (73%)

```
Query: 5   DIKPGXIQGCVGGRD 19
          D++PG ++G VG  D
Sbjct: 814 DVEPGIVEGSVGTED 828
```

>gi|34915974|dbj|BAC87876.1| Ricin B-related lectin [Polyporus squamosus]
Length = 292

Score = 22.3 bits (46), Expect = 6.5
Identities = 7/13 (53%), Positives = 8/13 (61%)

```
Query: 16 GGRDEGTPIIGWD 28
          G    GTP+I WD
Sbjct: 27 GSGQNGTPVIAWD 39
```

Figure 40. (Continued)

2-3-1 GTPIIGWDYXEX TFLADIKPGX IQGCVGGRDE

>gi|18476512|gb|AAL47680.1| agglutinin [Marasmius oreades]
Length = 293

Score = 23.1 bits (48), Expect = 3.8
Identities = 7/13 (53%), Positives = 9/13 (69%)

```
Query: 1  GTPIIGWDYXEXT 13
          GTPI+GW +   T
Sbjct: 29 GTPIVGWQFTPDT 41
```

>gi|7263614|emb|CAB81580.1| putative protein [Arabidopsis thaliana]
Length = 1113

Score = 22.3 bits (46), Expect = 6.5
Identities = 7/15 (46%), Positives = 11/15 (73%)

```
Query: 17  DIKPGXIQGCVGGRD 31
           D++PG ++G VG  D
Sbjct: 814 DVEPGIVEGSVGTED 828
```

3-2-1 TFLADIKPGX GTPIIGWDYXEX IQGCVGGRDE

>gi|18476512|gb|AAL47680.1| agglutinin [Marasmius oreades]
Length = 293

Score = 23.5 bits (49), Expect = 2.9
Identities = 9/18 (50%), Positives = 12/18 (66%), Gaps = 3/18 (16%)

```
Query: 5  DIKPGX---GTPIIGWDY 19
          D+K G    GTPI+GW +
Sbjct: 20 DLKDGSSSDGTPIVGWQF 37
```

1-3-2 IQGCVGGRDE TFLADIKPGX GTPIIGWDYXEX

>gi|18476512|gb|AAL47680.1| agglutinin [Marasmius oreades]
Length = 293

Score = 23.5 bits (49), Expect = 2.9
Identities = 9/18 (50%), Positives = 12/18 (66%), Gaps = 3/18 (16%)

```
Query: 15 DIKPGX---GTPIIGWDY 29
          D+K G    GTPI+GW +
Sbjct: 20 DLKDGSSSDGTPIVGWQF 37
```

Figure 40. (Continued)

NCBI DATABASE

No gapped sequence alignments of SCL

123 IQGCVGGRDEGTPIIGWDYXEXTFLADIKPGX
213 GTPIIGWDYXEXIQGCVGGRDETFLADIKPGX
312 TFLADIKPGXIQGCVGGRDEGTPIIGWDYXEX
231 GTPIIGWDYXEXTFLADIKPGXIQGCVGGRDE
321 TFLADIKPGXGTPIIGWDYXEXIQGCVGGRDE
132 IQGCVGGRDETFLADIKPGXGTPIIGWDYXEX

---

123 IQGCVGGRDEGTPIIGWDYXEXTFLADIKPGX

>gi|15238190|ref|NP_196615.1| lectin protein kinase, putative [Arabidopsis thaliana]
Length = 651

Score = 24.3 bits (51), Expect = 0.22
Identities = 9/24 (37%), Positives = 13/24 (54%)

```
Query: 6   GGRDEGTPIIGWDYXEXTFLADIK 29
           GG  EG  ++ W++     L DIK
Sbjct: 237 GGVTEGNRLLSWEFSSSLELIDIK 260
```

>gi|4115507|dbj|BAA36396.1| 29-kDa galactose-binding lectin [Lumbricus terrestris]
gi|4115505|dbj|BAA36395.1| 29-kDa galactose-binding lectin [Lumbricus terrestris]
Length = 260

Score = 21.2 bits (43), Expect = 1.9
Identities = 7/20 (35%), Positives = 9/20 (45%)

```
Query: 4   CVGGRDEGTPIIGWDYXEXT 23
           C G +D G  +  W Y   T
Sbjct: 101 CRGSKDVGAQVCAWKYHGGT 120
```

>gi|4115503|dbj|BAA36394.1| 29-kDa galactose-binding lectin [Lumbricus terrestris]
Length = 260

Score = 21.2 bits (43), Expect = 1.9
Identities = 7/20 (35%), Positives = 9/20 (45%)

```
Query: 4   CVGGRDEGTPIIGWDYXEXT 23
           C G +D G  +  W Y   T
Sbjct: 101 CRGSKDVGAQVCAWKYHGGT 120
```

>gi|4115501|dbj|BAA36393.1| 29-kDa galactose-binding lectin [Lumbricus terrestris]
Length = 260

Score = 21.2 bits (43), Expect = 1.9
Identities = 7/20 (35%), Positives = 9/20 (45%)

```
Query: 4   CVGGRDEGTPIIGWDYXEXT 23
           C G +D G  +  W Y   T
Sbjct: 101 CRGSKDVGAQVCAWKYHGGT 120
```

Figure 40. (Continued)

213 GTPIIGWDYXEXIQGCVGGRDETFLADIKPGX

>gi|18476512|gb|AAL47680.1| agglutinin [Marasmius oreades]
Length = 293

Score = 21.9 bits (45), Expect = 8.5
Identities = 6/9 (66%), Positives = 8/9 (88%)

```
Query: 1  GTPIIGWDY 9
          GTPI+GW +
Sbjct: 29 GTPIVGWQF 37
```

---

312 TFLADIKPGXIQGCVGGRDEGTPIIGWDYXEX

>gi|4115507|dbj|BAA36396.1| 29-kDa galactose-binding lectin [Lumbricus terrestris]
gi|4115505|dbj|BAA36395.1| 29-kDa galactose-binding lectin [Lumbricus terrestris]
Length = 260

Score = 20.8 bits (42), Expect = 2.5
Identities = 6/16 (37%), Positives = 8/16 (50%)

```
Query: 14  CVGGRDEGTPIIGWDY 29
           C G +D G  +  W Y
Sbjct: 101 CRGSKDVGAQVCAWKY 116
```

>gi|4115503|dbj|BAA36394.1| 29-kDa galactose-binding lectin [Lumbricus terrestris]
Length = 260

Score = 20.8 bits (42), Expect = 2.5
Identities = 6/16 (37%), Positives = 8/16 (50%)

```
Query: 14  CVGGRDEGTPIIGWDY 29
           C G +D G  +  W Y
Sbjct: 101 CRGSKDVGAQVCAWKY 116
```

>gi|4115501|dbj|BAA36393.1| 29-kDa galactose-binding lectin [Lumbricus terrestris]
Length = 260

Score = 20.8 bits (42), Expect = 2.5
Identities = 6/16 (37%), Positives = 8/16 (50%)

```
Query: 14  CVGGRDEGTPIIGWDY 29
           C G +D G  +  W Y
Sbjct: 101 CRGSKDVGAQVCAWKY 116
```

>gi|15238190|ref|NP_196615.1| lectin protein kinase, putative [Arabidopsis thaliana]
Length = 651

Score = 18.9 bits (37), Expect = 9.3
Identities = 5/14 (35%), Positives = 9/14 (64%)

```
Query: 16  GGRDEGTPIIGWDY 29
           GG  EG  ++ W++
Sbjct: 237 GGVTEGNRLLSWEF 250
```

Figure 40. (Continued)

231 GTPIIGWDYXEXTFLADIKPGXIQGCVGGRDE

>gi|15238190|ref|NP_196615.1| lectin protein kinase, putative [Arabidopsis thaliana]
Length = 651

Score = 19.2 bits (38), Expect = 7.1
Identities = 6/19 (31%), Positives = 10/19 (52%)

```
Query: 1   GTPIIGWDYXEXTFLADIK 19
           G  ++ W++     L DIK
Sbjct: 242 GNRLLSWEFSSSLELIDIK 260
```

---

321 TFLADIKPGXGTPIIGWDYXEXIQGCVGGRDE

>gi|18476512|gb|AAL47680.1| agglutinin [Marasmius oreades]
Length = 293

Score = 23.5 bits (49), Expect = 2.9
Identities = 9/18 (50%), Positives = 12/18 (66%), Gaps = 3/18 (16%)

```
Query: 5  DIKPGX---GTPIIGWDY 19
          D+K G    GTPI+GW +
Sbjct: 20 DLKDGSSSDGTPIVGWQF 37
```

---

132 IQGCVGGRDETFLADIKPGXGTPIIGWDYXEX

>gi|18476512|gb|AAL47680.1| agglutinin [Marasmius oreades]
Length = 293

Score = 23.5 bits (49), Expect = 2.9
Identities = 9/18 (50%), Positives = 12/18 (66%), Gaps = 3/18 (16%)

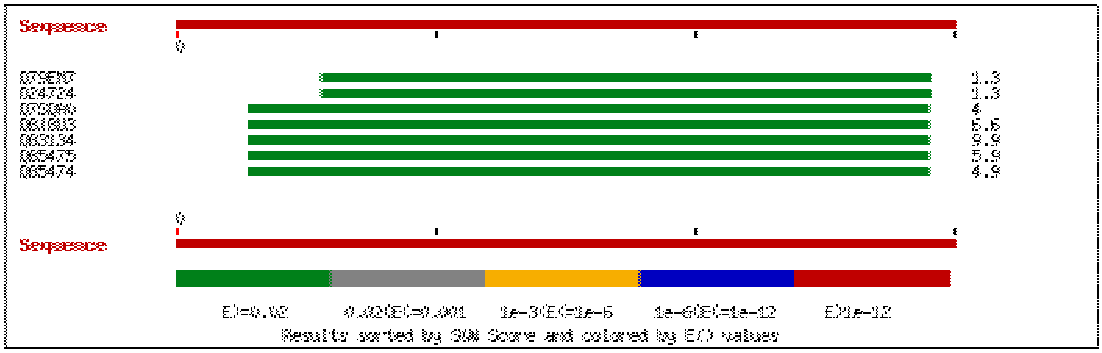
```
Query: 15 DIKPGX---GTPIIGWDY 29
          D+K G    GTPI+GW +
Sbjct: 20 DLKDGSSSDGTPIVGWQF 37
```

Figure 40. (Continued)

VISUALFASTA RESULTS FROM FASTA3 DATABASE

1-2-3 IQGCVGGRDE GTPIIGWDYXEX TFLADIKPGX
2-1-3 GTPIIGWDYXEX IQGCVGGRDE TFLADIKPGX
3-1-2 TFLADIKPGX IQGCVGGRDE GTPIIGWDYXEX
2-3-1 GTPIIGWDYXEX TFLADIKPGX IQGCVGGRDE
3-2-1 TFLADIKPGX GTPIIGWDYXEX IQGCVGGRDE
1-3-2 IQGCVGGRDE TFLADIKPGX GTPIIGWDYXEX

1-2-3 IQGCVGGRDE GTPIIGWDYXEX TFLADIKPGX




```
FASTA searches a protein or DNA sequence data bank
 version 3.4t23 March 18, 2004
Please cite:
 W.R. Pearson & D.J. Lipman PNAS (1988) 85:2444-2448

Query library @ vs +uniprot library
searching /ebi/services/idata/v987/fastadb/uniprot library

  1>>>Sequence - 32 aa
 vs  UniProt library

543565709 residues in 1696522 sequences
 statistics extrapolated from 60000 to 1696454 sequences
  Expectation_n fit: rho(ln(x))= 3.8121+/-0.000185; mu= 4.9474+/- 0.011
 mean_var=32.0313+/- 6.749, 0's: 444 Z-trim: 446  B-trim: 0 in 0/65
 Lambda= 0.226614
```

Figure 41. Results of sequence alignment of *Schizophyllum commune* lectin with other protein sequences from FASTA database.

```
FASTA (3.47 Mar 2004) function [optimized, BL50 matrix (15:-5)] ktup: 2
 join: 36, opt: 24, open/ext: -10/-2, width:  16
 Scan time: 114.450
The best scores are:                                      opt bits E(1696522)
UNIPROT:O24724 O24724 2-carboxybenzaldehyde dehyd  ( 485)   88  34.2
1.3
UNIPROT:Q79EM7 Q79EM7 2-carboxybenzaldehyde dehyd  ( 485)   88  34.2
1.3
UNIPROT:Q7SQA0 Q7SQA0 Envelope protein.            ( 611)   84  32.9
4
UNIPROT:Q85474 Q85474 Envelope protein (Fragment)  ( 271)   80  31.5
4.9
UNIPROT:Q85475 Q85475 Envelope protein (Fragment)  ( 335)   80  31.5
5.9
UNIPROT:Q8J8U3 Q8J8U3 Envelope protein (Fragment)  ( 495)   81  31.9
6.6
UNIPROT:Q83134 Q83134 Env protein.                 ( 607)   80  31.6
9.9

>>UNIPROT:O24724 O24724 2-carboxybenzaldehyde dehydrogen  (485 aa)
 initn:  51 init1:  51 opt:  88  Z-score: 155.1  bits: 34.2 E():  1.3
Smith-Waterman score: 88;  53.846% identity (56.000% ungapped) in 26 aa
overlap (6-31:357-381)

                                      10        20        30
Sequen                             IQGCVGGRDEGTPIIGWDYXEXTFLADIKPGX
                                      ::: ::.   :: :   : :::.::
UNIPRO MGPLVSQAQYDKSVHAIGEGIREGAKVVAGGGRPEGVGEGGW-YLAPTVLADVRPGSFIE
        330       340       350       360       370       380

UNIPRO QNEIFGPVLSVIIFATDDEAVAIANGVEYGLTASVWTSDITRAHLIARRVEAGYVLVNGG
         390       400       410       420       430       440

>>UNIPROT:Q79EM7 Q79EM7 2-carboxybenzaldehyde dehydrogen  (485 aa)
 initn:  51 init1:  51 opt:  88  Z-score: 155.1  bits: 34.2 E():  1.3
Smith-Waterman score: 88;  53.846% identity (56.000% ungapped) in 26 aa
overlap (6-31:357-381)

                                      10        20        30
Sequen                             IQGCVGGRDEGTPIIGWDYXEXTFLADIKPGX
                                      ::: ::.   :: :   : :::.::
UNIPRO MGPLVSQAQYDKSVHAIGEGIREGAKVVAGGGRPEGVGEGGW-YLAPTVLADVRPGSFIE
        330       340       350       360       370       380

UNIPRO QNEIFGPVLSVIIFATDDEAVAIANGVEYGLTASVWTSDITRAHLIARRVEAGYVLVNGG
         390       400       410       420       430       440

>>UNIPROT:Q7SQA0 Q7SQA0 Envelope protein.                (611 aa)
 initn:  53 init1:  53 opt:  84  Z-score: 146.5  bits: 32.9 E():    4
Smith-Waterman score: 84;  38.710% identity (41.379% ungapped) in 31 aa
overlap (3-31:190-220)
```

Figure 41. (Continued).

```
                                          10          20        30
Sequen                                IQGCVGGRDEGTP--IIGWDYXEXTFLADIKP
                                        ::.:    :.:  . :::  : : .    :
UNIPRO LLDEPSELQLLGSQSLPNITNITQIPSVAGGCIGFTPYGSPAGVYGWDRREVTHILLTDP
     160       170       180       190       200       210


Sequen GX
       :
UNIPRO GSNPFFDKASKSSKPFTVVTADRHNLFMGSEYCGAYGYRFWEMYNCSQMRQWSICMDVWG
     220       230       240       250       260       270
```

>>UNIPROT:Q85474 Q85474 Envelope protein (Fragment). (271 aa)
 initn: 49 init1: 49 opt: 80 Z-score: 144.9 bits: 31.5 E(): 4.9
Smith-Waterman score: 80; 35.484% identity (37.931% ungapped) in 31 aa overlap (3-31:121-151)

```
                                          10          20        30
Sequen                                IQGCVGGRDEGTP--IIGWDYXEXTFLADIKP
                                        ::.:    :.:  . :::  . : .    :
UNIPRO LLDEPSELQLLGSQSLPNITNITRIPSVAGGCIGFTPYGSPAGVYGWDRRQVTHILLTDP
              100       110       120       130       140       150


Sequen GX
       :
UNIPRO GNNPFFDKASNSSKPFTVVTADRHNLFMGSEYCGAYGYRFWEIYNCSHRFDNFDIYTCGD
              160       170       180       190       200       210
```

>>UNIPROT:Q85475 Q85475 Envelope protein (Fragment). (335 aa)
 initn: 49 init1: 49 opt: 80 Z-score: 143.4 bits: 31.5 E(): 5.9
Smith-Waterman score: 80; 35.484% identity (37.931% ungapped) in 31 aa overlap (3-31:121-151)

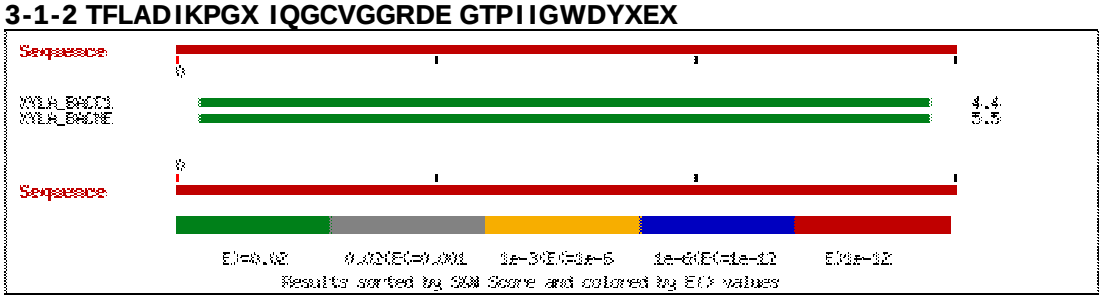
```
                                          10          20        30
Sequen                                IQGCVGGRDEGTP--IIGWDYXEXTFLADIKP
                                        ::.:    :.:  . :::  . : .    :
UNIPRO LLDEPSELQLLGSQSLPNITNITRIPSVAGGCIGFTPYGSPAGVYGWDRRQVTHILLTDP
              100       110       120       130       140       150


Sequen GX
       :
UNIPRO GNNPFFDKASNSSKPFTVVTADRHNLFMGSEYCGAYGYRFWEMYNCSQMRQNWSICQDVW
              160       170       180       190       200       210
```

>>UNIPROT:Q8J8U3 Q8J8U3 Envelope protein (Fragment). (495 aa)
 initn: 50 init1: 50 opt: 81 Z-score: 142.6 bits: 31.9 E(): 6.6
Smith-Waterman score: 81; 35.484% identity (37.931% ungapped) in 31 aa overlap (3-31:192-222)

Figure 41. (Continued).

```
                                          10          20        30
Sequen                                IQGCVGGRDEGTP--IIGWDYXEXTFLADIKP
                                        ::.:    :.:  . :::  . : .   .:
UNIPRO LLDEPSELQLLGSQSLPNITNITWIPSVAGGCIGFTPYGSPAGVYGWDRRQVTHILLTNP
             170       180       190       200       210       220


Sequen GX
       :
UNIPRO GSNPFFDKASNSSKPFTVVTADRHNLFMGSEYCGAYGYRFWEMYNCSQYPQYPNWSVCQD
             230       240       250       260       270       280

>>UNIPROT:Q83134 Q83134 Env protein.                        (607 aa)
 initn:  49 init1:  49 opt:  80  Z-score: 139.4  bits: 31.6 E():  9.9
Smith-Waterman score: 80;  35.484% identity (37.931% ungapped) in 31 aa
overlap (3-31:191-221)

                                          10          20        30
Sequen                                IQGCVGGRDEGTP--IIGWDYXEXTFLADIKP
                                        ::.:    :.:  . :::  . : .    :
UNIPRO LLDEPSELQLLGSQSLPNITDITRIPTVAGGCIGFTPYGSPAGVYGWDRRQVTHILLTDP
              170       180       190       200       210       220


Sequen GX
       :
UNIPRO GNNPFFDKASNSSKPFTVVTADRHNLFMGSEYCGAYGYRFWEMYNCSQMSQNWSICQDVW
              230       240       250       260       270       280



32 residues in 1 query   sequences
543565709 residues in 1696522 library sequences
 Tcomplib [34t23] (4 proc)
 start: Fri Dec 10 08:41:31 2004 done: Fri Dec 10 08:42:41 2004
 Total Scan time: 114.450 Total Display time:  0.000

Function used was FASTA [version 3.4t23 March 18, 2004]
```

3-1-2 TFLADIKPGX IQGCVGGRDE GTPIIGWDYXEX




```
FASTA searches a protein or DNA sequence data bank
 version 3.4t23 March 18, 2004
Please cite:
 W.R. Pearson & D.J. Lipman PNAS (1988) 85:2444-2448
```

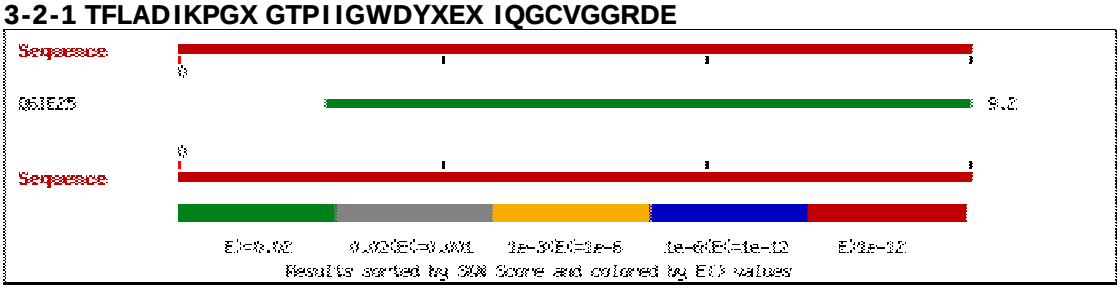
Figure 41. (Continued).

```
Query library @ vs +uniprot library
searching /ebi/services/idata/v987/fastadb/uniprot library

  1>>>Sequence - 32 aa
 vs  UniProt library

543565709 residues in 1696522 sequences
 statistics extrapolated from 60000 to 1696493 sequences
  Expectation_n fit: rho(ln(x))= 4.0645+/-0.00019; mu= 4.0756+/- 0.011
 mean_var=35.6090+/- 7.498, 0's: 444 Z-trim: 445  B-trim: 610 in 1/64
 Lambda= 0.214929

FASTA (3.47 Mar 2004) function [optimized, BL50 matrix (15:-5)] ktup: 2
 join: 36, opt: 24, open/ext: -10/-2, width:  16
 Scan time: 172.116
The best scores are:                                      opt bits E(1696522)
 UNIPROT:XYLA_BACC1 Q739D2 Xylose isomerase (EC 5.  ( 445)   86  32.3
4.4
 UNIPROT:XYLA_BACME O08325 Xylose isomerase (EC 5.  ( 445)   85  32.0
5.5

>>UNIPROT:XYLA_BACC1 Q739D2 Xylose isomerase (EC 5.3.1.5  (445 aa)
 initn:  42 init1:  42 opt:  86  Z-score: 145.8  bits: 32.3 E():  4.4
Smith-Waterman score: 86;  36.364% identity (38.710% ungapped) in 33 aa
overlap (1-31:284-316)

                                          10          20
Sequen                                TFLADIKPGXIQGCVGGRD--EGTPIIGWD
                                      ::  ... . .:: .:. :  .:.:..:::
UNIPRO ATTISFLRQYGLENHFKLNLEANHATLAGHTFEHELRVARVQGFLGSVDANQGNPLLGWD
           260       270       280       290       300       310

       30
Sequen YXEX
         :
UNIPRO TDEFPTNLYSTTLAMYEILQNGGLGSGGLNFDAKVRRASFEEEDLVYAHIAGMDAFARGL
           320       330       340       350       360       370

>>UNIPROT:XYLA_BACME O08325 Xylose isomerase (EC 5.3.1.5  (445 aa)
 initn:  42 init1:  42 opt:  85  Z-score: 144.1  bits: 32.0 E():  5.5
Smith-Waterman score: 85;  36.364% identity (38.710% ungapped) in 33 aa
overlap (1-31:284-316)

                                          10          20
Sequen                                TFLADIKPGXIQGCVGGRD--EGTPIIGWD
                                      ::  ... . .:: .:. :  .: :..:::
UNIPRO ATTISFLRQYGLDKYFKLNLEANHATLAGHTFEHELRVARVQGLLGSVDANQGDPLLGWD
           260       270       280       290       300       310

       30
Sequen YXEX
         :
UNIPRO TDEFPTDLYSTTLAMYEILQNGGLGSGGLNFDAKVRRGSFEQDDLLYAHVAGMDAFARGL
           320       330       340       350       360       370
```

Figure 41. (Continued).

```
32 residues in 1 query   sequences
543565709 residues in 1696522 library sequences
 Tcomplib [34t23] (4 proc)
 start: Fri Dec 10 09:19:12 2004 done: Fri Dec 10 09:20:34 2004
 Total Scan time: 172.116 Total Display time:  0.000

Function used was FASTA [version 3.4t23 March 18, 200
```

3-2-1 TFLADIKPGX GTPIIGWDYXEX IQGCVGGRDE




```
FASTA searches a protein or DNA sequence data bank
 version 3.4t23 March 18, 2004
Please cite:
 W.R. Pearson & D.J. Lipman PNAS (1988) 85:2444-2448

Query library @ vs +uniprot library
searching /ebi/services/idata/v987/fastadb/uniprot library

  1>>>Sequence - 32 aa
 vs  UniProt library

543565709 residues in 1696522 sequences
 statistics extrapolated from 60000 to 1696505 sequences
  Expectation_n fit: rho(ln(x))= 3.8630+/-0.000187; mu= 4.5539+/- 0.011
 mean_var=32.7128+/- 7.166, 0's: 457 Z-trim: 458  B-trim: 2036 in 1/64
 Lambda= 0.224241

FASTA (3.47 Mar 2004) function [optimized, BL50 matrix (15:-5)] ktup: 2
 join: 36, opt: 24, open/ext: -10/-2, width:  16
 Scan time: 162.633
The best scores are:                                      opt bits E(1696522)
 UNIPROT:Q6IE25 Q6IE25 Caspase 14-like protein.     ( 176)   76  29.9
9.2

>>UNIPROT:Q6IE25 Q6IE25 Caspase 14-like protein.          (176 aa)
 initn: 103 init1:  50 opt:  76  Z-score: 140.0  bits: 29.9 E():  9.2
Smith-Waterman score: 76;  44.444% identity (54.545% ungapped) in 27 aa
overlap (6-32:93-114)
```

Figure 41. (Continued).

```
10        20        30
Sequen                           TFLADIKPGXGTPIIGWDYXEXIQGCVGGRDE
                                      ..:: :: ..:     .:.:::. :::
UNIPRO RHWLRAPRAIPTQADVLQIHADAPGGSAFLLEPGLGTQVLG-----TIHGCVAYRDEKGS
            70        80        90       100            110

UNIPRO DFVQTLVEVIRANPGRDLLELLTEVNRRVCELDVLGPDSDELCKACLEIRSLLRRRLCL
       120       130       140       150       160       170


32 residues in 1 query   sequences
543565709 residues in 1696522 library sequences
 Tcomplib [34t23] (4 proc)
 start: Fri Dec 10 09:46:30 2004 done: Fri Dec 10 09:47:48 2004
 Total Scan time: 162.633 Total Display time:  0.000

Function used was FASTA [version 3.4t23 March 18, 2004
```

Figure 41. (Continued)

APPENDIX H

LECTIN CRYSTALLIZATION AND X-RAY CRYSTALLOGRAPHY

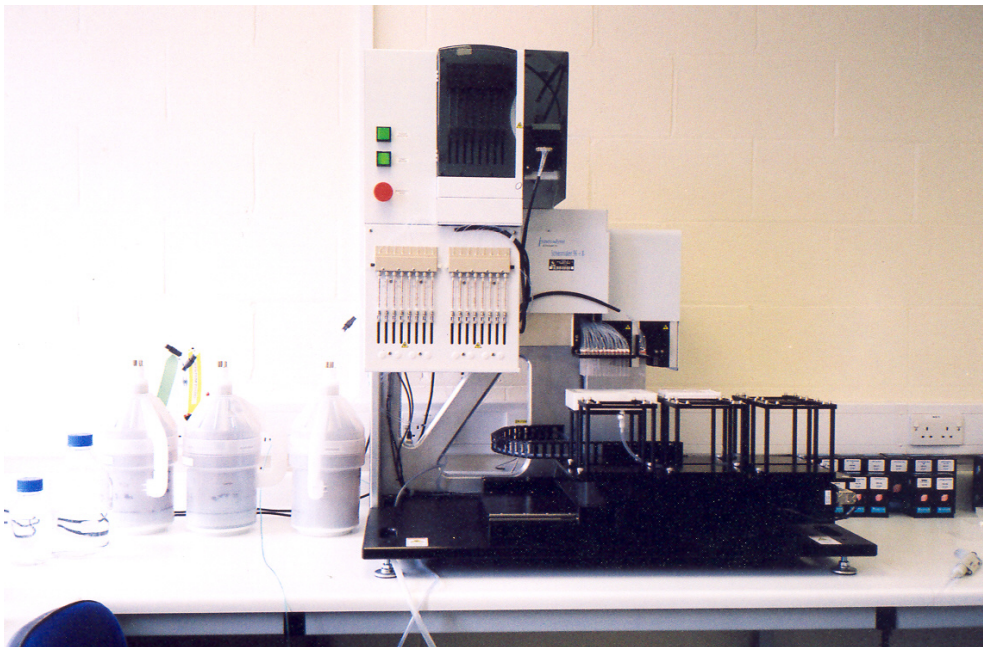


Figure 42. Crystallization robot located at Daresbury laboratory, Warrington, Cheshire, U.K.

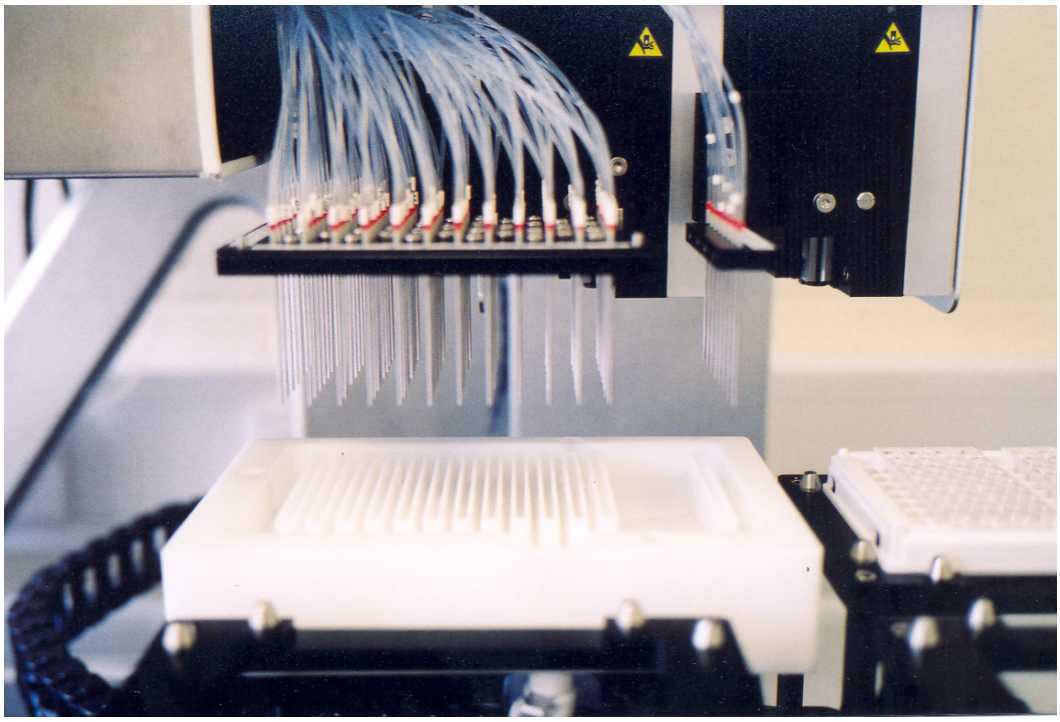


Figure 43. The crystallization robot focusing on the injection tubes with built-in the machine.

Figure 44. Crystal Pro machine using for protein crystal growth monitoring.

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
B	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
C	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
D	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
E	E1	E2	E3	E4	E5	E6	E7	E8	D9	E10	D11	D12
F	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
G	G1	G2	G3	G4	G5	G6	G7	G8	**G9**	**G10**	G11	G12
H	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12

Figure 45. Sprase matrix of lectin crystallization by crystallization robot via sitting drop technique

Table 14. Observations in a sitting drop crystallization experiment using JBScreen HTS I *Formulations* (PEG based) reagent kit (Jena Bioscience, CA).

Grid and buffer conditions	Incubation time	
	1 week	2 weeks
A1 15% PEG 400, 0.1 M Na Acetate pH 4.6, 0.1 M Ca Chloride	1	1
A2 15% PEG 400, 0.1 M Na HEPES pH 7.5, 0.2 M Mg Chloride	1	1
A3 25% PEG 400, 0.1 M Na Acetate pH 4.6, 0.1 M Mg Chloride	5	5
A4 25% PEG 400, 0.1 M TRIS HCl pH 8.5, 0.2 M Li Sulfate	3	3
A5 30% PEG 400, 0.1 M Na Acetate pH 4.6, 0.1 M Ca Chloride	4	4
A6 30% PEG 400, 0.1 M Na MES pH 6.5, 0.1 M Na Acetate	4	4
A7 30% PEG 400, 0.1 M Na HEPES pH 7.5, 0.2 M Mg Chloride	3	3
A8 30% PEG 400, 0.1 M TRIS HCl pH 8.5, 0.2 M Na Citrate	1	1
A9 30% PEG 550 MME, 0.1 M Bicine pH 9.0, 0.1 M Na Chloride	1	1
A10 25% PEG 550 MME, 0.1 M Na MES pH 6.5, 0.01 M Zn Sulfate	1	3
A11 25% PEG 1000, 0.1 M Na HEPES pH 7.5	1	5
A12 30% PEG 1000, 0.1 M TRIS HCl pH 8.5*	1	7
B1 15% PEG 1500	1	2
B2 20% PEG 1500, 0.1 M Na HEPES pH 7.5	1	2
B3 30% PEG 1500*	1	5
B4 20% PEG 2000 MME, 0.1 M TRIS HCl pH 8.5, 0.01 M Ni Chloride	1	1
B5 25% PEG 2000 MME*	1	4
B6 20% PEG 3000, 0.1 M Na HEPES pH 7.5, 0.2 M Na Acetate*	1	4
B7 30% PEG 3000, 0.1 M TRIS HCl pH 8.5, 0.2 M Li Sulfate	1	4
B8 4% PEG 4000, 0.1 M Na Acetate pH 4.6	1	1
B9 8% PEG 4000	1	1
B10 8% PEG 4000, 0.1 M Na Acetate pH 4.6	1	1
B11 10% PEG 4000, 0.1 M Na MES pH 6.5, 0.2 M Mg Chloride	1	1
B12 12% PEG 4000, 0.1 M Na HEPES pH 7.5, 0.1 M Na Acetate	1	1
C1 16% PEG 4000, 0.1 M TRIS HCl pH 8.5, 0.2 M Li Sulfate	5	5
C2 16% PEG 4000, 0.1 M TRIS HCl pH 8.5, 0.2 M Na Acetate	1	1
C3 18% PEG 4000, 0.1 M Na Acetate pH 4.6	1	1
C4 20% PEG 4000, 0.1 M TRIS HCl pH 8.5, 0.2 M Li Sulfate	3	4
C5 20% PEG 4000, 0.1 M TRIS HCl pH 8.5, 0.2 M Ca Chloride*	6	6
C6 25% PEG 4000, 0.1 M Na Acetate pH 4.6*	3	4
C7 25% PEG 4000, 0.1 M Na MES pH 6.5, 0.2 M Mg Chloride	3	3
C8 25% PEG 4000, 0.1 M TRIS HCl pH 8.5, 0.2 M Ca Chloride*	6	4
C9 30% PEG 4000	3	3
C10 30% PEG 4000, 0.1 M Na Acetate pH 4.6, 0.1 M Mg Chloride	3	3
C11 30% PEG 4000, 0.1 M Na HEPES pH 7.5, 0.2 M Ca Chloride	4	4
C12 30% PEG 4000, 0.1 M TRIS HCl pH 8.5, 0.2 M Na Acetate*	4	4

Table 14. (Continued)

Grid and Buffer conditions	Incubated Time	
	1 week	2 weeks
D1 30% PEG 4000, 0.1 M TRIS HCl pH 8.5, 0.2 M Mg Chloride*	1	4
D2 35% PEG 4000	1	3
D3 8% PEG 4000, 0.8 M Li Chloride, 0.1 M TRIS HCl pH 8.5	1	1
D4 10% PEG 4000, 20% *iso*-Propanol	1	1
D5 10% PEG 4000, 10% *iso*-Propanol, 0.1 M Na Citrate pH 5.	1	4
D6 10% PEG 4000, 20% *iso*-Propanol, 0.1 M Na HEPES pH 7.5	1	1
D7 12% PEG 4000, 0.1 M Na Acetate pH 4.6, 0.2 M Ammonium Sulfate	1	1
D8 15% PEG 4000, 0.1 M Na Citrate pH 5.6, 0.2 M Ammonium Sulfate	1	4
D9 16% PEG 4000, 10% *iso*-Propanol, 0.1 M Na HEPES pH 7.5, 0.2 M NH_2SO_4*	1	8
D10 20% PEG 4000, 0.2 M Ammonium Sulfate*	1	8
D11 20% PEG 4000, 10% Glycerol, 0.2 M Mg Sulfate	4	4
D12 20% PEG 4000, 20% *iso*-Propanol, 0.1 M Na Citrate	4	1
E1 20% PEG 4000, 0.6 M Na Chloride, 0.1 M Na MES pH 6.5*	7	7
E2 20% PEG 4000, 10% *iso*-Propanol, 0.1 M Na HEPES pH 7.5*	3	5
E3 22% PEG 4000, 0.2 M Ammonium Sulfate, 0.1 M Na Acetate*	7	7
E4 25% PEG 4000, 0.1 M Na Citrate pH 5.6, 0.2 M Ammonium Sulfate*	4	4
E5 25% PEG 4000, 0.1 M Na HEPES pH 7.5, 0.2 M Li Sulfate, 0.1 M Na Acetate*	4	4
E6 25% PEG 4000, 8% *iso*-Propanol, 0.1 M Na Acetate*	3	3
E7 30% PEG 4000, 0.2 M Ammonium Sulfate	3	1
E8 30% PEG 4000, 0.1 M Na Citrate pH 5.6, 0.2 M Ammonium Sulfate	3	1
E9 32% PEG 4000, 0.8 M Li Chloride 0.1 M TRIS HCl pH 8.5	1	1
E10 25% PEG 5000 MME, 0.1 M TRIS HCl pH 8.5, 0.2 M Li Sulfate	3	1
E11 30% PEG 5000 MME, 0.1 M Na MES pH 6.5, 0.2 M Ammonium Sulfate	3	1
E12 3% PEG 6000, 0.1 M TRIS HCl pH 8.5, 0.1 M K Chloride	1	1
F1 10% PEG 6000, 0.01 M Mg Chloride	1	1
F2 12% PEG 6000, 2.0 M Na Chloride	1	1
F3 15% PEG 6000, 5% Glycerol	1	1
F4 15% PEG 6000, 0.05 M K Chloride, 0.01 M Mg Chloride*	1	7
F5 20% PEG 6000, 0.05 M Imidazole HCl pH 8.0*	4	4
F6 25% PEG 6000, 0.1 M Na HEPES pH 7.5, 0.1 M Li Chloride	3	3
F7 28% PEG 6000, 0.5 M Li Chloride, 0.1 M TRIS HCl pH 8.5	1	1
F8 30% PEG 6000, 1 M Li Chloride, 0.1 M Na Acetate	1	1
F9 2% PEG 8000, 0.5 M Li Sulfate	3	1
F10 2% PEG 8000, 1.0 M Li Sulfate	3	1
F11 4% PEG 8000	3	1
F12 8% PEG 8000, 0.2 M Li Chloride, 0.05 M Mg Sulfate	2	3

Table 14. (Continued)

Grid and Buffer conditions	Incubated Time	
	1 week	2 weeks
G1 10% PEG 8000, 0.1 M Na MES pH 6.5, 0.2 M Zn Acetate	1	1
G2 10% PEG 8000, 0.1 M Na HEPES pH 7.5, 0.2 M Ca Acetate	1	1
G3 10% PEG 8000, 0.05 M Mg Acetate, 0.1 M Na Acetate	1	1
G4 10% PEG 8000, 0.2 M Mg Acetate	1	1
G5 10% PEG 8000, 10% Ethylene Glycol, 0.1 M Na HEPES pH 7.5	3	4
G6 12% PEG 8000, 10% Glycerol, 0.5 M K Chloride	3	3
G7 15% PEG 8000, 0.2 M Ammonium Sulfate*	5	5
G8 15% PEG 8000, 0.5 M Li Sulfate*	6	6
G9 15% PEG 8000, 0.1 M Na MES pH 6.5, 0.2 M Na Acetate*	8	8
G10 18% PEG 8000, 0.1 M Na HEPES pH 7.5, 0.2 M Ca Acetate*	8	8
G11 18% PEG 8000, 2% *iso*-Propanol, 0.1 M Na HEPES pH 7.5, 0.1 MNa Acetate	4	4
G12 18% PEG 8000, 0.1 M TRIS HCl pH 8.5, 0.2 M Li Sulfate	3	3
H1 20% PEG 8000, 0.1 M Na MES pH 6.5, 0.2 M Mg Acetate	1	1
H2 20% PEG 8000, 0.1 M CHES pH 9.5	1	1
H3 25% PEG 8000, 0.2 M Li Chloride	1	1
H4 30% PEG 8000, 0.2 M Ammonium Sulfate	1	1
H5 8% PEG 10000, 0.1 M Na Acetate pH 4.6	1	1
H6 14% PEG 10000, 0.1 M Imidazole HCl pH 8.0	1	1
H7 18% PEG 10000, 20% Glycerol, 0.1 M TRIS HCl pH 8.5, 0.1 M Na Chloride	3	3
H8 20% PEG 10000, 0.1 M Na HEPES pH 7.5	3	3
H9 30% PEG 10000, 0.1 M TRIS HCl pH 8.5	1	1
H10 10% PEG 20000, 0.1 M Na MES pH 6.5	4	4
H11 17% PEG 20000, 0.1 M TRIS HCl pH 8.5, 0.1 M Mg Chloride	1	1
H12 20% PEG 20000	1	1

Note 1= Clear drop, 2= Phase seperation, 3= Regular granular precipitate, 4= Birefringent or microcrystals, 5=Posettes or spherulites, 6=Needles (1D growth), 7=Plates (2D growth), 8= Single crystals (3D growth<0.2 mm)
* Crystal that was submitted to next step of x-ray diffraction experiments, Li= Lithium, Na= Sodium

JBScreen HTS II *Formulations* Cat. No.: CS-202 (Jena Bioscience, CA) (Ammonium Sulfate, MPD, Alcohol and Salt based)

A1 0.5 M Ammonium Sulfate, 1.0 M Li Sulfate, 0.1M Na Citrate
A2 1.0 M Ammonium Sulfate, 0.1 M Na Acetate pH 4.6
A3 1.0 M Ammonium Sulfate, 2% PEG 400, 0.1 M Na HEPES pH 7.5
A4 1.0 M Ammonium Sulfate, 0.1 M TRIS HCl pH 8.5
A5 1.2 M Ammonium Sulfate, 3% *iso*-Propanol, 0.05 M Na Citrate
A6 1.5 M Ammonium Sulfate, 15% Glycerol, 0.1 M TRIS HCl pH 8.5
A7 1.6 M Ammonium Sulfate, 1.0 M Li Sulfate
A8 1.6 M Ammonium Sulfate, 0.1 M Na HEPES pH 7.5, 0.2 M Na Chloride
A9 1.6 M Ammonium Sulfate, 2% PEG 1000, 0.1 M Na HEPES pH 7.5
A10 1.8 M Ammonium Sulfate, 0.1 M Na MES pH 6.5
A11 2.0 M Ammonium Sulfate, 2 M Na Chloride
A12 2.0 M Ammonium Sulfate, 0.1 M Na Acetate pH 4.6

B1 2.0 M Ammonium Sulfate, 5% PEG 400, 0.1 M Na MES pH 6.5
B2 2.0 M Ammonium Sulfate, 0.1 M TRIS HCl pH 8.5
B3 2.2 M Ammonium Sulfate, 20% Glycerol
B4 2.4 M Ammonium Sulfate, 0.1 M Na Citrate
B5 3.0 M Ammonium Sulfate, 1% MPD
B6 3.0 M Ammonium Sulfate, 10% Glycerol
B7 3.5 M Ammonium Sulfate, 0.1 M Na HEPES pH 7.5
B8 3.5 M Ammonium Sulfate, 1% MPD, 0.1 M Na MES pH 6.5
B9 10% MPD, 0.1 M Na HEPES pH 7.5, 0.1 M Na Citrate
B10 12% MPD, 0.1 M TRIS HCl pH 8.5, 0.05 M Mg Chloride
B11 15% MPD, 5% PEG 4000, 0.1 M Imidazole HCl pH 8.0
B12 15% MPD, 0.1 M Na Citrate pH 5.6, 0.2 M Ammonium Acetate

C1 15% MPD, 0.1 M Na HEPES pH 7.5, 0.2 M Na Citrate
C2 20% MPD, 0.1 M Na HEPES pH 7.5, 0.1 M Na Citrate
C3 20% MPD, 0.1 M Imidazole HCl pH 8.0
C4 20% MPD, 4% Glycerol, 0.2 M Na Chloride
C5 30% MPD, 0.1 M Na Acetate pH 4.6, 0.02 M Ca Chloride
C6 30% MPD, 0.1 M Na Citrate pH 5.6, 0.2 M Ammonium Acetate
C7 30% MPD, 0.5 M Ammonium Sulfate, 0.1 M Na HEPES pH 7.5
C8 30% MPD, 0.1 M Na HEPES pH 7.5, 0.2 M Na Citrate
C9 30% MPD, 10% PEG 4000, 0.1 M Imidazole HCl pH 8.0
C10 30% MPD, 20% Ethanol
C11 35% MPD, 0.1 M Imidazole HCl pH 8.0
C12 40% MPD, 0.1 M TRIS HCl pH 8.5

D1 47% MPD, 0.1 M Na HEPES pH 7.5
D2 47% MPD, 2% *tert.*-Butanol
D3 50% MPD
D4 50% MPD, 20% *iso*-Propanol, 0.05 M Na Acetate, 0.05 M Na Chloride
D5 50% MPD, 0.1 M TRIS HCl pH 8.5, 0.2 M Ammonium Dihydrogen Phosphate
D6 55% MPD
D7 60% MPD, 0.1 M Na Acetate pH 4.6, 0.01 M Ca Chloride
D8 70% MPD, 0.1 M Na MES pH 6.5
D9 70% MPD, 0.1 M TRIS HCl pH 8.5
D10 20% Methanol, 0.1 M TRIS HCl pH 8.5, 0.01 M Ca Chloride
D11 2% Ethanol, 0.1 M TRIS HCl pH 8.5
D12 5% Ethanol, 5% MPD, 0.1 M TRIS HCl pH 8.5, 0.2 M Na Chloride

E1 10% Ethanol, 0.1 M TRIS HCl pH 8.5
E2 12% Ethanol, 4% PEG 400, 0.1 M Na Acetate pH 4.6
E3 14% Ethanol, 5% Glycerol, 0.1 M TRIS HCl pH 8.5
E4 18% Ethanol, 0.1 M TRIS HCl pH 8.5
E5 20% Ethanol, 10% Glycerol
E6 30% Ethanol, 10% PEG 6000, 0.1 M Na Acetate
E7 45% Ethanol
E8 50% Ethanol, 0.01 M Na Acetate
E9 60% Ethanol, 1.5% PEG 6000, 0.05 M Na Acetate
E10 60% Ethanol, 0.1 M Na Chloride
E11 5% *iso*-Propanol, 0.1 M Na HEPES pH 7.5
E12 10% *iso*-Propanol, 0.1 M Na Acetate pH 4.6, 0.2 M Ca Chloride

F1 10% *iso*-Propanol, 0.1 M Na HEPES pH 7.5, 0.2 M Na Citrate
F2 10% *iso*-Propanol, 0.1 M TRIS HCl pH 8.5, 0.01 M Mg Chloride
F3 15% *iso*-Propanol, 0.1 M Na MES pH 6.5, 0.2 M Na Citrate
F4 15% *iso*-Propanol, 0.1 M Na HEPES pH 7.5, 0.2 M Mg Chloride
F5 15% *iso*-Propanol, 0.1 M TRIS HCl pH 8.5, 0.2 M Ammonium Acetate
F6 20% *iso*-Propanol, 0.1 M Na Acetate pH 4.6, 0.2 M Ca Chloride
F7 25% *iso*-Propanol, 0.1 M Na HEPES pH 7.5, 0.1 M Mg Chloride
F8 30% *iso*-Propanol, 0.1 M Na MES pH 6.5, 0.2 M Na Citrate
F9 30% *iso*-Propanol, 0.1 M TRIS HCl pH 8.5, 0.2 M Ammonium Acetate
F10 25% *tert.*-Butanol, 0.1 M TRIS HCl pH 8.5, 0.1 M Ca Chloride
F11 35% *tert.*-Butanol, 0.1 M Na Citrate pH 5.6
F12 0.2 M Ammonium Dihydrogen Phosphate

G1 0.2 M K,Na Tartrate
G2 0.2 M Mg Acetate
G3 0.4 M Ammonium Dihydrogen Phosphate
G4 0.4 M K,Na Tartrate, 0.1 M TRIS HCl pH 8.5
G5 0.5 M Ammonium Dihydrogen Phosphate, 0.2 M Na Citrate
G6 0.5 M Na Acetate, 0.1 M Imidazole HCl pH 8.0
G7 0.7 M Na Citrate, 0.1 M Na HEPES pH 7.5
G8 0.8 M K,Na Tartrate, 0.1 M Na HEPES pH 7.5
G9 1.0 M Ammonium Dihydrogen Phosphate, 0.1 M Na Citrate pH 5.6
G10 1.0 M Ammonium Dihydrogen Phosphate, 0.1 M TRIS HCl pH 8.5
G11 1.0 M Li Sulfate, 0.1 M TRIS HCl pH 8.5, 0.01 M Ni Chloride
G12 1.0 M Na Acetate, 0.1 M Imidazole HCl pH 8.0

H1 1.4 M Na Acetate, 0.1 M Na MES pH 6.5
H2 1.5 M Li Sulfate, 0.1 M TRIS HCl pH 8.5
H3 1.5 M Na Citrate pH 6.5
H4 1.6 M Mg Sulfate, 0.1 M Na MES pH 6.5
H5 1.6 M K,Na Tartrate, 0.1 M Na MES pH 6.5
H6 2.0 M Ammonium Formate, 0.1 M Na MES pH 6.5
H7 2.0 M Ammonium Dihydrogen Phosphate, 0.1 M TRIS HCl pH 8.5
H8 2.0 M Na Chloride, 0.1 M Na MES pH 6.5, 0.2 M Na Acetate
H9 2.0 M Na Formate, 0.1 M Na Acetate pH 4.6
H10 1.0 M Ammonium Dihydrogen Phosphate, 30% Glycerol, 0.1 M TRIS HCl pH 8.5
H11 4.0 M Na Chloride, 0.1 M Na HEPES pH 7.5
H12 3.0 M Na Formate

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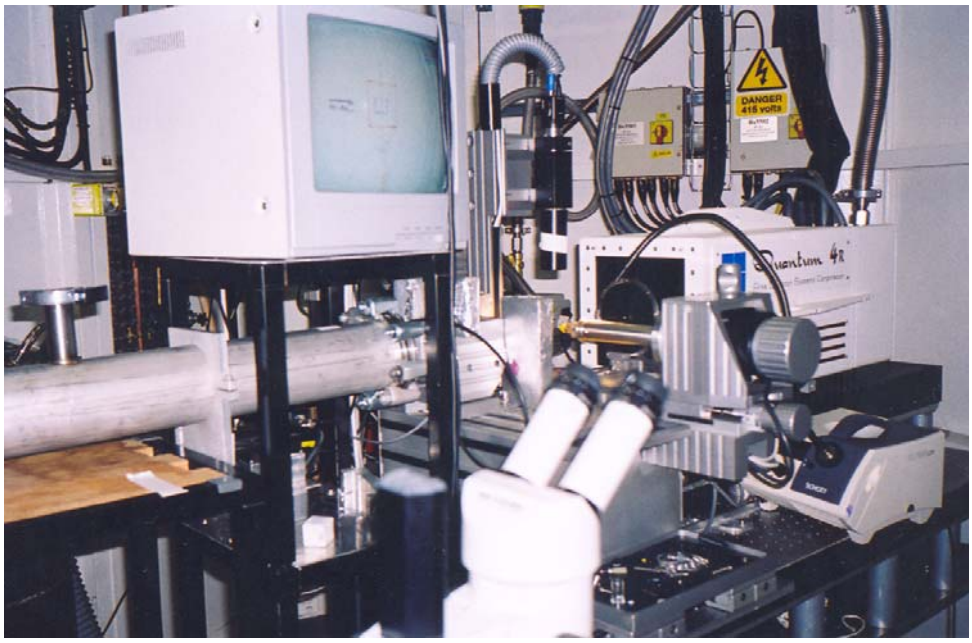

Figure 46. A typical Synchrotron data collection station 14.1 at Daresbury, Cheshire, U.K. (1) a charge-coupled device (CCD) detector, (2) The nozzle which stream of nitrogen at 100K, (3) the spindle which can rotate the crystal manually to ensure perfect alignment, (4) the X-ray beamline runs tangentially from the storage ring.

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