LECTINS FROM STRAW MUSHROOM CULTIVATED IN NORTH-EASTERN THAILAND

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เล็กตินจากเห็ดฟางเพาะเลี้ยงในภาคตะวันออกเฉียงเหนือของประเทศไทย

นางสาวนฤมล โม้ทอง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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LECTINS FROM STRAW MUSHROOM CULTIVATED IN NORTH-EASTERN THAILAND

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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นฤมล โม้ทอง : เล็กตินจากเห็ดฟางเพาะเลี้ยงในภาคตะวันออกเฉียงเหนือของประเทศไทย (LECTINS FROM STRAW MUSHROOM CULTIVATED IN NORTH-EASTERN THAILAND) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.สุรีลักษณ์ รอดทอง, 193 หน้า.

เล็กตินเป็นสารโปรตีนหรือไกลโคโปรตีนที่มีความจำเพาะคล้ายแอนติบอดีที่สามารถทำให้เกิดการจับกลุ่มของเซลล์ และได้จากแหล่งผลิตที่ไม่เกี่ยวข้องกับระบบภูมิคุ้มกัน การศึกษาครั้งนี้เพื่อตรวจหา ทำบริสุทธิ์ ศึกษาคุณลักษณะ และปลูกผลึกสารเล็กตินจากเห็ดฟางเพาะเลี้ยงในภาคตะวันออกเฉียงเหนือของประเทศไทย จากการตรวจหาสารเล็กตินที่สะสมในตัวอย่างดอกเห็ดฟางจำนวน 11 ตัวอย่าง ด้วยปฏิกิริยาการจับกลุ่มของเซลล์เม็ดเลือดแดงของกระต่าย พบว่า เห็ดฟางทุกตัวอย่างสะสมสารเล็กตินในปริมาณแตกต่างกันทั้งที่แยกส่วนหมวก ส่วนก้าน และรวมทุกส่วนของดอกเห็ด ตามค่าความเจือจางสูงสุดของสารสกัดหยาบเล็กตินเริ่มต้นที่ความเจือจาง 1:10 เกิดการจับกลุ่มเซลล์เม็ดเลือดแดงของกระต่ายที่ความเจือจางสูงสุดโดยเฉลี่ยเท่ากับ 2,048 2,048 และ 4,096 ตามลำดับ เมื่อนำสารสกัดหยาบเล็กตินที่ได้ไปทดสอบความสามารถในการยับยั้งการเจริญของจุลินทรีย์ พบสารเล็กตินเพียง 2 ตัวอย่าง คือ รหัส MC131 และ MC133 มีฤทธิ์ในการยับยั้งการเจริญของ *Bacillus subtilis* ATCC 6633 *Staphylococcus aureus* ATCC 29213 และ *Escherichia coli* ATCC 25922 ที่ความเข้มข้น 0.090 และ 0.123 0.057 และ 0.053 และ 0.116 และ 0.128 ไมโครกรัม/ไมโครลิตร ตามลำดับ เมื่อเทียบกับสารสเตรปโตมัยซินมาตรฐาน สารสกัดหยาบเล็กตินรหัส MC134 และ MC145 ยับยั้ง *Staphylococcus aureus* ATCC 29213 ที่ความเข้มข้น 0.066 และ 0.065 ไมโครกรัม/ไมโครลิตร ตามลำดับ สารเล็กตินรหัส MC145 และ MC168 สามารถยับยั้ง *Aspergillus niger* ATCC 6275 ได้โดยมีความกว้างของบริเวณยับยั้งน้อยกว่าสารไนสเตตินมาตรฐาน (100 หน่วย) 1.65 และ 1.46 เท่า ตามลำดับ สาร MC168 ยังสามารถยับยั้ง *Candida albican* ATCC 10231 และ *Penicillium funiculosum* ATCC 36839 ได้โดยมีความกว้างของบริเวณยับยั้งน้อยกว่าและมากกว่าสารไนสเตตินมาตรฐาน (100 หน่วย) 0.60 และ 1.38 เท่า ตามลำดับ เมื่อทดสอบความเป็นพิษของสารเล็กตินต่อเซลล์มะเร็งเยื่อบุช่องปากและเซลล์มะเร็งปากมดลูกของคนที่เพาะเลี้ยง พบว่า สารสกัดหยาบเล็กติน MC131 สามารถยับยั้งการเพิ่มจำนวนเซลล์มะเร็งเยื่อบุช่องปากได้ครึ่งหนึ่งเมื่อเทียบกับเซลล์ควบคุม (IC_{50}) ที่ค่าความเข้มข้นเท่ากับ 4.80 ไมโครกรัม/มิลลิลิตร และมีเพียงสาร MC134 ที่สามารถยับยั้งการเพิ่มจำนวนเซลล์มะเร็งปากมดลูกได้ครึ่งหนึ่งเมื่อเทียบกับเซลล์ควบคุมที่ค่าความเข้มข้นเท่ากับ 21.38 ไมโครกรัม/มิลลิลิตร จึงเลือกเห็ดฟางรหัส MC131 ซึ่งมีลำดับนิวคลีโอไทด์ของ 18S rRNA gene ขนาด 1,687 คู่เบส เหมือนมากที่สุดที่ร้อยละ 98 กับ *Volvariella volvacea* JM leg. SLR (DQ851588) ตามฐานข้อมูล GenBank สหรัฐอเมริกา มาสกัดแยกสารเล็กตินเพื่อทำบริสุทธิ์ จากการศึกษาพบว่า วิธีที่มีประสิทธิภาพในการทำบริสุทธิ์

สารเล็กตินของเห็ดฟางคือ การตกตะกอนสารเล็กตินด้วยเกลือแอมโมเนียมซัลเฟตร้อยละ 90 จากนั้นแยกสารโดยการแลกเปลี่ยนประจุ และแยกตามขนาดโมเลกุลด้วยเจลฟิวเตรชันโครมาโทกราฟี (Gel filtration chromatography) สารเล็กตินบริสุทธิ์ MC131 เป็นสารไกลโคโปรตีน มีน้ำหนักโมเลกุล 25 กิโลดาลตัน มีความเหมือนของลำดับกรดอะมิโนร้อยละ 99 กับโปรตีน Volvatoxin A2 precursor ที่มี ขนาด 24.2 กิโลดาลตัน ของ *Volvariella volvacea* (AAQ92757.1) ตามฐานข้อมูล GenBank มีจุดที่มีประจุสุทธิเป็นศูนย์ (พีไอ) ประมาณ 5 และไม่มีความจำเพาะต่อน้ำตาลชนิดใดเลยจากน้ำตาล 32 ชนิดที่ทดสอบ สารเล็กตินบริสุทธิ์ MC131 ทนอุณหภูมิสูงสุดที่ 60 องศาเซลเซียส เป็นเวลา 30 นาที และเสถียรที่ค่าความเป็นกรด-ด่าง 7.0 ตลอดระยะเวลา 18 ชั่วโมงที่ทดสอบ ในขณะที่สารสกัดหยาบเล็กตินจากเห็ดตัวอย่างนี้ทนอุณหภูมิสูงสุดที่ 60 องศาเซลเซียส เป็นเวลา 1 ชั่วโมง และเสถียรต่อค่าความเป็นกรด-ด่างที่ 5-12 ตลอดระยะเวลา 18 ชั่วโมงที่ทดสอบ สารเล็กตินบริสุทธิ์ MC131 ไม่มีฤทธิ์ยับยั้งจุลินทรีย์ชนิดที่สารสกัดหยาบยับยั้งได้ดังกล่าวข้างต้น แต่สามารถยับยั้งการเพิ่มจำนวนเซลล์มะเร็งเยื่อบุช่องปากและเซลล์มะเร็งปากมดลูกของคนที่เพาะเลี้ยงได้ครึ่งหนึ่งเมื่อเทียบกับเซลล์ควบคุมที่ค่าความเข้มข้นเท่ากับ 0.58 และ 0.70 ไมโครกรัม/มิลลิลิตร ตามลำดับ เมื่อนำสารเล็กตินบริสุทธิ์ MC131 มาทดลองปลูกผลึกพบสภาวะที่เหมาะสมทำให้ได้ผลึกเล็กตินที่มีลักษณะเพลทที่ให้แบบแผนการกระจายรังสีเอกซ์ที่มีการแจกแจงรายละเอียด 3 อังสตรอม ข้อมูลที่ได้จากการศึกษาเป็นประโยชน์ต่อการศึกษาสารเล็กตินและโครงสร้างของสารในเชิงลึกต่อไป กล่าวโดยสรุปได้ว่า เห็ดฟางเพาะเลี้ยงในที่ต่างๆ ในภาคตะวันออกเฉียงเหนือของประเทศไทย เป็นแหล่งของสารเล็กตินที่มีสมบัติทางเคมี และชีวภาพด้านความสามารถในการยับยั้งการเจริญของจุลินทรีย์ และความเป็นพิษต่อเซลล์มะเร็งของคนได้แตกต่างกัน จึงมีแนวโน้มที่จะประยุกต์ใช้ประโยชน์ทางเภสัชกรรม การแพทย์ และการวิจัยทางวิทยาศาสตร์

สาขาวิชาจุลชีววิทยา
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ลายมือชื่อนักศึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____
ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_____

NARUMOL MOTHONG : LECTINS FROM STRAW MUSHROOM CULTIVATED IN NORTH-EASTERN THAILAND. THESIS ADVISOR : ASST. PROF. SUREELAK RODTONG, Ph.D. 192 PP.

LECTIN / STRAW MUSHROOM / *Volvariella volvacea* / PURIFICATION / CRYSTALLIZATION

Lectins able to agglutinate cells, are proteins or glycoproteins of non-immune origin. Investigation of lectins from straw mushroom cultivated in North-eastern Thailand, were performed. All 11 straw mushroom specimens collected from different locations, were found to accumulate lectins in caps, stalks and whole fruiting bodies at different hemagglutination titers (from 1:10 original lectin dilution and using rabbit red blood cells) on the average of 2,048, 2,048 and 4,096, respectively. The crude extracts were then tested for their biological properties. Lectins from specimen codes MC131 and MC133 inhibited *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922 at concentrations of 0.090 and 0.123, 0.057 and 0.053, and 0.116 and 0.128 μg/μl compared to standard streptomycin, respectively. MC134 and MC145 extracts inhibited *S. aureus* ATCC 29213 at concentrations of 0.066 and 0.065 μg/μl, respectively. MC145 and MC168 extracts could inhibit *Aspergillus niger* ATCC 6275 at 1.65 and 1.46 times, respectively, narrower than standard nystatin (100 U) inhibition zone diameter. MC168 extract also inhibited *Candida albican* ATCC 10231 and *Penicillium funiculosum* ATCC 36839 at 0.60 and 1.38 times narrower and wider than standard nystatin inhibition zone, respectively. For cytotoxic test against two human cancer

cells, crude MC131 lectin extract displayed strong cytotoxicity against human epidermoid carcinoma (KB) cells with 50% inhibition concentration (IC_{50}) value of 4.80 μg/ml. Only MC134 extract showed cytotoxicity against human cervical carcinoma (HeLa) cells (IC_{50} of 21.38 μg/ml). The mushroom MC131 having 98% nucleotide sequence similarity of 18S rRNA gene (1,687 bp) compared to *Volvariella volvacea* (DQ851588) from GenBank database, U.S.A., was then selected for lectin purification. MC131 lectin could be purified by 90% ammonium sulfate precipitation, ion exchange and gel filtration chromatography. The lectin was proven to be a 25 kDa glycoprotein, that showed 99% amino acid sequence similarity compared to 24.2 kDa of *V. volvacea* volvatoxin A2 precursor (AAQ92757.1), and had isoelectric point (pI) of approximately 5. The purified lectin was not specific to 32 sugars tested, but stable at 60°C for 30 min and at pH 7 for 18 h test whereas the crude extract was stable for 1 h and at pH 5-12. The glycoprotein exhibited no antimicrobial activity but the crude extract showed cytotoxicity against KB and HeLa cells (IC_{50} of 0.58 and 0.70 μg/ml, respectively). The plate-like crystals of MC131 lectin giving an X-ray diffraction pattern of 3.0 Å resolution, were achieved. Results obtained reveal that straw mushroom cultivated in North-eastern Thailand, could be served as a source of lectins possessing biological properties that will be useful for further study and applications.

School of Microbiology

Academic Year 2009

Student's Signature_____

Advisor's Signature_____

Co-advisor's Signature_____

Co-advisor's Signature_____

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LIST OF ABBREVIATIONS

Å	Angstrom
A_{280}	Absorbance at 280
A_{595}	Absorbance at 595
AIDS	Acquired immune deficiency syndrome
ATCC	American Type Culture Collection
bp	Base pair
BSA	Bovine serum albumin
$^{\circ}C$	Degree celsius
CCD	Charge-coupled device
CFU	Colony forming unit
CRD	Carbohydrate recognition domain
cm	Centimeter
CM	Carboxymethyl
ConA	Concanavalin A
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dNTPs	Deoxynucleoside triphosphate
dTTP	Deoxythymidine triphosphate
DEAE	Diethylaminoethyl
DMSO	Dimethyl sulfoxid

LIST OF ABBREVIATIONS (Continued)

DNA	Deoxyribonucleic acid
et al.	et alia (and others)
e.g.	For example
etc.	et cetera, and others
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic recticulum
FBS	Fetal bovine serum
g	Gram
Gal	Galactose
GalNAc	*N*-Acetyl-D-galactosamine
h	Hour
HA	Influenza virus hemagglutination
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
HeLa	Human cervical carcinoma
IC_{50}	50% inhibitory concentration
K	Kelvin
Kb	Kilobase
KB	Human epidermoid carcinoma
kDa	Kilodaltons
l	Liter
LC/ESI/MS	Liquid chromatography-electrospray ionization mass spectrometry

LIST OF ABBREVIATIONS (Continued)

M	Molar
MEM	Minimum essential medium
MHA	Mueller Hinton agar
min	Minute
mg	Milligram
ml	Milliliter
mm	Millimeter
mM	Millimolar
MS	Mass spectrometry
MW	Molecular weight
NCBI	National Center for Biotechnology Information
ng	Nanogram
nm	Nanometer
nt	Nucleotide
%	Percentage
PAS	Periodic acid Schiff
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pp	Page
PVPP	Polyvinylpyrrolidone
RBCs	Red blood cells

LIST OF ABBREVIATIONS (Continued)

RIPs	Ribosome inactivating proteins
rRNA	Ribosomal ribonucleic acid
RNA	Ribonucleic acid
RNase B	Ribonuclease B
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
sec	Second
SLRI	Synchrotron Light Research Institute (Public Organization)
TCA	Trichloroacetic acid
TMED	*N,N,N′,N′*-Tetramethylethylenediamine
TMV	Tobacco mosaic virus
µg	Microgram
µl	Microliter
µm	Micrometer
U.K.	United Kingdom
U.S.A.	United States of America
UV	Ultraviolet
v/v	Volume by volume
Vero	African green monkey kidney epithelial
w/v	Weight by volume

CHAPTER I

INTRODUCTION

1.1 Significance of the study

Lectins are proteins or glycoproteins of non-immune origin that specifically and reversibly bind to carbohydrates of glycoconjugates (Rini, 1995). These proteins are widely distributed in nature, and occur in diverse organisms ranging from plants, animals, fungi, bacteria and viruses (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). Because of their carbohydrate recognition, lectins are used in a number of immunological and biochemical fields (Lis and Sharon, 1998). Several of them have been commercially utilized (Wang *et al*., 1998). In recent years, there has been a growing interest in mushroom lectins, largely due to the discovery that some of these lectins exhibit a series of distinct bioactivities. For example, *Agaricus bisporus* lectin possesses antiproliferative activity against human colon cancer cell line (HT29) and breast cancer cell line (MCF-7) (Yu *et al*., 1999), *Schizophyllum commune* lectin is cytotoxic to human epidermoid carcinoma cell line (KB) (Chumkhunthod *et al*., 2006). *Armillaria luteo-virens* lectin shows inhibition activity to proliferation of tumor cells including mouse T cell leukemia (MBL2), human cervical carcinoma cell line (HeLa) and mouse leukemia cell line (L1210) (Feng *et al*., 2006). Lectins from *Agrocybe cylindracea* (Wang *et al*., 2002), *Boletus satanas* Lenz, *Ganoderma lucidum*, *Flammulina*

velutipes and *Volvariella volvacea* (Ho *et al*., 2004) exhibit the potent mitogenic activity toward mouse lymphocytes. In addition, some mushroom lectins possess other potential activities such as HIV-1 reverse transcriptase inhibitory, immunoenhancing, antimicrobial and anti-insect activities (Zheng *et al*., 2007). These clearly indicate that mushrooms have been becoming a valuable source of lectins for drug discovery or therapeutic agents for medical and pharmaceutical applications.

Thailand, a tropical country, is considered to be one of the most potential countries for mushroom lectin investigation. One hundred and sixty eight mushroom specimens collected from natural habitats in various locations in Thailand, were found to accumulate lectins (Chumkhunthod, 2004). Some of the lectins exhibit biological activities. Straw mushroom, *Volvariella volvacea*, the edible mushroom, is widely cultivated in Thailand, and has been reported to accumulate lectins in its fruiting bodies (Wang *et al*., 1998). The mushroom lectins were preliminary tested for their cytotoxicity in our laboratory.

In this study, lectins from straw mushrooms cultivated in North-eastern Thailand, were intensively determined for their biological activities. The straw mushroom lectin with potent antimicrobial activity and cytotoxicity was purified and characterized. The information on the straw mushroom lectins will be useful for further investigation and applications.

1.2 Research objectives

The objectives of this research were:

1) To detect the accumulation of lectins in fruiting bodies of straw mushroom cultivated in North-eastern Thailand.

2) To purify and characterize the straw mushroom lectins for their chemical, physical and biological properties for further investigation and applications.

3) To crystallize the purified straw mushroom lectins in order to investigate the chemical structure.

1.3 Research hypothesis

Lectins accumulated in straw mushroom, cultivated in North-eastern Thailand, having biological activity, could be detected. Purification and characterization of the straw mushroom lectins could provide informations of both known and novel lectins. Straw mushroom lectins could be useful for medical and pharmaceutical applications.

1.4 Scope and limitations of the study

Fresh fruiting bodies of straw mushroom specimens cultivated in North-eastern Thailand were collected from local markets in North-eastern Thailand. Straw mushroom specimens were confirmed for their identification relied on morphological characteristics Lectins accumulated in straw mushroom specimens were detected by hemagglutination assay. Physical and biological properties of crude lectin extracts were determined. The selected straw mushroom lectin was purified by

chromatography and characterized the chemical, physical and biological properties of its lectin. The purified straw mushroom lectin was preliminary crystallized.

1.5 Expected results

From this study, the information of lectin accumulations in fruiting bodies of straw mushroom cultivated in North-eastern Thailand was obtained. The suitable purification technique, chemical, physical and biological property data of the straw mushroom lectins were achieved for further investigation and applications. The crystallization conditions of straw mushroom lectins were obtained. The conditions were beneficial for studying three dimensional structure which could be applied for specific recognition mechanism investigation of the mushroom lectins.

CHAPTER II

LITERATURE REVIEW

2.1 Lectins

Lectins are proteins or glycoproteins of non-immune origin which are able to agglutinate cells through sugar-specific binding sites, and to precipitate polysaccharides and glycoconjugates (Rini, 1995). Both agglutination and precipitation reactions of lectins are inhibited by sugar ligands for which the lectins are specific. Each lectin molecule commonly contains one or more (typically two) carbohydrate-combining sites which are referred to carbohydrate recognition domains (CRD) (Rini, 1995). However, some lectins possess only one binding site for their specific sugars, therefore they cannot agglutinate red blood cells (Sharon and Lis, 1989). Lectins may interact with carbohydrates through hydrogen bonds, metal coordination, Van der Waals and hydrophobic interactions. Generally, hydroxyl groups on sugar molecules can serve as both a donor and an acceptor to cooperate in hydrogen bonds (Elgavish and Shaanan, 1997). Hemagglutinating activity is a major attribute of lectins, and is widely used for their detection and characterization (Lis and Sharon, 1998).

Lectins are synthesized at the endoplasmic recticulum (ER) of eukaryotic cells and consequently modified at the Golgi complex apparatus by glycosylational process. Several lectins have been described in the calnexin-calreticulin cycle (Figure 2.1) (Helenius and Aebi, 2001).

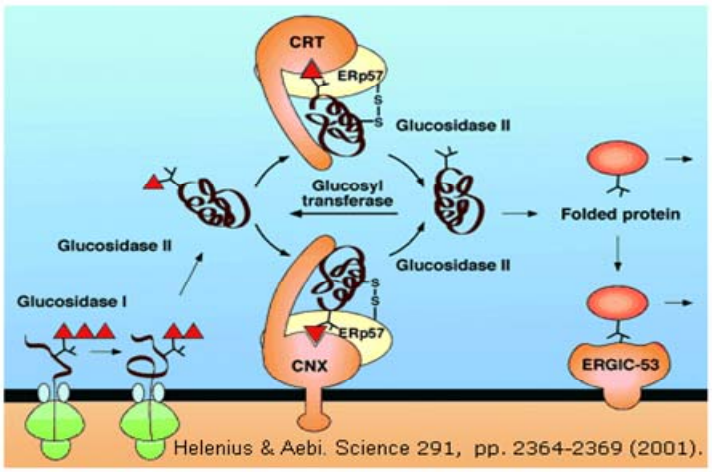


Figure 2.1 The calnexin-calreticulin cycle.

Source: Helenius and Aebi (2001)

The carbohydrates are linked to the protein component through either O-glycosidic or N-glycosidic bonds. The N-glycosidic linkage links through the amide group of asparagines, whereas the O-glycosidic linkage links through the hydroxyl of serine, threonine or hydroxylysine. When two of the glucose residues in the N-linked core glycans have been trimmed away by glucosidases I and II, the nascent or newly synthesized glycoproteins bind to calnexin (CNX) and/or calreticulin (CRT). If the glycoproteins have cysteines, the formation of disulfide bonds is catalyzed through the formation of transient mixed disulfides with ERp57, another folding factor. When the remaining third glucose is trimmed bvhy glucosidase II, the complexes dissociate. If the glycoprotein is not folded at this time, the oligosaccharides are reglucosylated by an ER-glucosyltransferase, and the protein reassociates with the lectins. The cycle is repeated until the protein is either folded or degraded. Once folded correctly, the protein is no longer recognized by the glucosyltransferase, and because it is no longer

reglucosylated, it will not bind back to calnexin and/or calreticulin. It can now leave the ER. Exit of certain glycoproteins from the ER to the Golgi complex is assisted by ERGIC-53, another membrane- bound lectin that combines with mannose residues.

2.1.1 Structures of lectins

Most lectins are divided into three classes according to their structural features: simple, mosaic (or multidomain) and macromolecular assemblies (Lis and Sharon, 1998). Within each class, lectins can be grouped into distinct families with similar sequences and structural properties. Simple lectins consist of a small number subunits, not necessarily identical, each of molecular weight usually below 40 kDa, which may contains a carbohydrate-binding site. This class comprises practically all known plant lectins as well as the galectins (S-type lectins) from animals and mushrooms, *Coprinus cinereus* (Cooper *et al*., 1997) and *Agrocybe cylindracea* (Ban *et al*., 2005).

Mosaic lectins are composite molecules with a wide range of molecular weights, consisting of several kinds of protein domains, only one of which possesses a carbohydrate-binding site. This class includes diverse proteins from different sources; hemagglutinins from viruses and the C-, P- and I-type lectins from animals.

Macromolecular assemblies are common in bacteria, usually in the form of fimbriae (or pili). They are filamentous organelles consisting of helically arranged protein subunits (pilins) assembled in a well-defined order. Only one of the subunits, usually a minor component of the fimbriae, possesses a carbohydrate combining site, and is responsible for the binding activity and sugar specificity of the fimbriae.

2.1.2 Sources of lectins

Lectins are widely distributed in living organisms including animals, plants and microorganisms (bacteria, fungi, algae and viruses).

2.1.2.1 Animals

Animal lectins have been found in vertebrates including mammals as well as invertebrates, such as snails, crabs, worms (Dodd and Drickamer, 2001), insects (Kubo *et al*., 2001), mollusks and sponges (Müller *et al*., 1997). These lectins are classified on the basis of shared sequence characteristics of their carbohydrate recognition domains (CRDs) (Dodd and Drickamer, 2001). The major types are C-type lectins (a superfamily) and S-type lectins (galectins).

C-type lectins are Ca^{2+}dependent animal lectins that are carbohydrate-binding proteins of animal origin (Drickamer, 1999). They are associated both with the plasma membrane and intracellular membranes. C-type lectins can be categorized into two types: collectins and selectins. Collectins are soluble C-type lectins secreted from cells. They can participate in host defense mechanism, for example, through complement activation, e.g., serum mannose binding protein (MBP). Selectins are transmembrane C-type lectins. They are known as a family of cell-cell adhesion molecules that are involved in the adhesive interaction between leukocytes and vascular endothelial cells, which is required for extravasation at target tissue sites.

S-type lectins or galectins are soluble, β-galactose specific lectins and metal-independent in their activity. They are localization in nuclei, on cell surfaces and in extracellular spaces, depending on the galectin species. Galectins are found in both vertebrates and invertebrates, and having even been found in the

mushrooms *Coprinus cinereus* (Cooper *et al*., 1997) and *Agrocybe cylindracea* (Ban *et al*., 2005). Their functions are related to development, differentiation, morphogenesis, tumor metastasis, apoptosis and RNA splicing. Most galectins contain multiple sugar-binding sites, and have no disulfide bridges, no sugar chains, no signal sequences, and in most cases their N-terminal amino acids are acetylated.

2.1.2.2 Plants

Plant lectins are a heterogeneous group of carbohydrate-binding proteins. They can be classified into four groups of evolutionarily related proteins, where are legume lectins, chitin-binding lectins, type 2 ribosome-inactivating proteins and monocot mannose-binding lectins (Van Damme *et al*., 1997).

Legume lectins are a large family of homologous carbohydrate-binding proteins which are confined to species of the plant family Leguminoseae (Sharon and Lis, 1990). All legume lectins contain divalent cations at specific metal-binding sites. Each subunit possesses both Mn^{2+} and Ca^{2+} ions, which are held in place by interactions with specific amino acid residues, and are essential for the carbohydrate binding activity of the lectins. They have been identified in seeds, leaves, stems, bark and roots of legume plants (Imberty *et al*., 2000). The best known legume lectins are concanavalin A from jack bean, phytohemagglutinin (PHA) from red kidney bean, soybean (SBA), peanut (PNA) and favin from fava bean (Lis and Sharon, 1998).

Chitin-binding lectins with hevein domains have been isolated from several plant families such as Gramineae, Urticaceae, Solanaceae, Papaveraceae, Euphorbiaceae, Phytolaccaceae and Viscaceae (Peumans *et al*., 1996). The best

known chitin-binding lectins with hevein domains are wheatgerm agglutinin (WGA), pokeweed mitogen, rice, rye and barley lectins (Wood *et al*., 1999). Most of these lectins also react with GlcNAc, GlcNAc-oligomers and *N*-acetyl-D-neuraminic acid.

Type 2 ribosome-inactivating proteins (Type 2 RIPs) consist of an A chain and B chain, where the A chain (a toxic subunit) possesses RNA N-glycosidase activity, causing the inactivation of the ribosome and inhibition of protein synthesis, and the B chain (Gal/GalNAc-binding subunit) is responsible for binding of the whole protein molecule to the target-cell surface and also for helping the A chain to cross the cell membrane (Wood *et al*., 1999). The best known examples of this family are ricin from seeds of *Ricinus communis* (Robert *et al*., 1985) and abrin from seeds of *Abrus precatorius* (Wood *et al*., 1991).

Monocot mannose-binding lectins are an extended superfamily of evolutionarily related proteins that preferably bind to 1–3- or 1–6-linked D-mannoses with the highest affinity (Van Damme *et al*., 1988). They are found to present three potential carbohydrate binding motifs per subunit, each of which contains a consensus sequence signature QXDXNXVXY, essential for mannose binding (Ramachandraiah *et al*., 2000). The first monocot mannose-binding lectin was reported from the snowdrop (*Galanthus nivalis*) bulbs (Van Damme *et al*., 1988). Later, several lectins have been isolated and characterized from several monocot families, such as Amaryllidaceae, Alliaceae, Araceae, Orchidaceae and Liliaceae (Barre *et al*., 1996).

2.1.2.3 Microorganisms

A) Bacteria

Many bacterial species express lectins, more than one type and with distinct specificities. In Gram-negative bacteria, such as *Escherichia coli*, *Klebsiella pneumoniae* and *Salmonellae* sp., the lectins often are in the form of fimbriae (or pili). Fimbrial surface lectins are also produced by Gram-positive bacteria. Non-fimbrial lectins associated with the bacterial surface have been purified from *Rhizobium lupinii* and *Agrobacterium tumefaciens* (Ofek and Doyle, 1994).

B) Fungi

Filamentous fungi and mushrooms have been reported to produce lectins. High contents of lectins in fungi are detected in diverse species of genera belonging to basidiomycetes, such as *Agaricus, Agrocybe, Amanita, Boletus, Gymnopilus, Hebeloma, Hygrophorus, Lactarius, Mycena, Phallus, Pleurotus* and *Russula* (Wang *et al*., 1998). The mushroom lectins have been purified from fruiting bodies of *Agaricus campestris* and *Agaricus bisporus* (Goldstein and Poretz, 1986). A galectin, the first of lectin family found outside the animal kingdom has been isolated from the fruit bodies of *Coprinus cinereus* (Cooper *et al*., 1997). However, several lectins have been reported from ascomycetes, *Aleuria aurantia* (Kochibe *et al*., 1980), *Melastiza chateri* (Ogawa *et al*., 2001), *Ciborinia camelliae* (Otta *et al*., 2002), *Sclerotinia sclerotiorum* (Candy *et al*., 2003), *Xylaria hypoxylon* (Liu *et al*., 2006) and *Cordyceps militaris* (Jung *et al*., 2007). A few yeast species, such as *Saccharomyces cerevisiae* (Kundu *et al*., 1987) and *Kluyveromyces bulgaricus* (Al-Mahmood *et al*., 1991) can produce lectins. The parasitic fungus, *Arthrobotrys*

oligospora, posseses a multispecific lectin that can bind to fetuin and mucins (Rosen *et al*., 1996).

C) Algae

Alga lectins have been isolated and characterized. Mori *et al*. (2005) reported that griffithsin, a lectin from the red alga *Griffithsia* sp., displayed potent antiviral activity against laboratory stains and primary isolates of macrophage (M-tropic) and T-cell (T-tropic) strains of HIV-1. In addition, some lectins found in cyanobacteria, such as cyanovirin-N from *Nostoc ellipsosporum* (Boyd *et al*., 1997), scytovirin from *Scytonema varium* (Bokesch *et al*., 2003), *Microcystis viridis* lectin from *Microcystis viridis* (Yamaguchi *et al*., 1999), exhibit significant activity against human immunodeficiency virus (HIV) which makes them particularly promising targets for the development as novel antiviral agents.

D) Viruses

Viruses contain sugar-specific surface proteins or glycoproteins that act as hemagglutinins, and are classified as lectins (Sharon and Lis, 1997). Much information is available on the influenza and polyoma viruses belonging to the orthomyxoviruses and papoviruses, respectively. Similar lectins that are less well defined, are found in myxoviruses. Other viral lectins include those of HIV (Haidar *et al*., 1992) and herpes simplex (Spillmann, 2001).

2.2 Mushrooms

Mushrooms are higher fungi belonging to class Basidiomycetes in phylum Basidiomycota of the kingdom Fungi that produce conspicuous fruit bodies (Alexopoulos and Mims, 1996). This class produces externally basidiospores on the sterigmata of basidia in basidiocarps during sexual reproduction. The cell walls are usually chitin. Many species produce clamp connections, swellings that are associated with septa of the dikaryotic mycelium. Several mushrooms degrade dead organic matters, or act as parasites on living organisms. Others form beneficial structure to plants as mycorrhizae. Mushrooms play essential roles in the ecological balance of the environment, i.e., transport, store, release and recycle nutrients because of their ability in breaking down the dead materials. Many mushrooms are confined to a very specific environment for growing and forming fruit bodies. Their habitats and climate are major factors that indicate their diversity (Nilsson and Presson, 1978a; 1978b).

The fruiting body of mushrooms is usually composed of a stalk (stipe), a hymenium, a cap (pileus), spores, ring or annulus (some species) and volva (some species). It is also multiform in its shape and coloring (Figure 2.2).

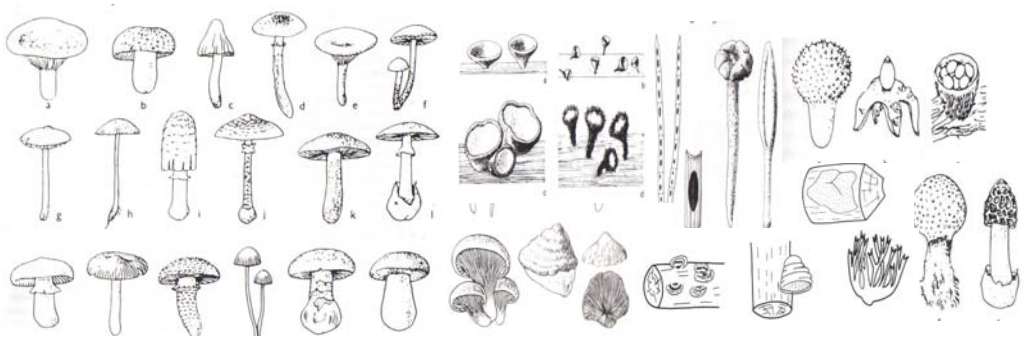


Figure 2.2 Various shapes of fruiting bodies of mushrooms.

Source: Svrček (2000)

A cap (pileus) is borne on a stalk. It can be various shapes: the most familiar being hemispherical or convex, with many of this becoming flat as it mature (Figure 2.3).

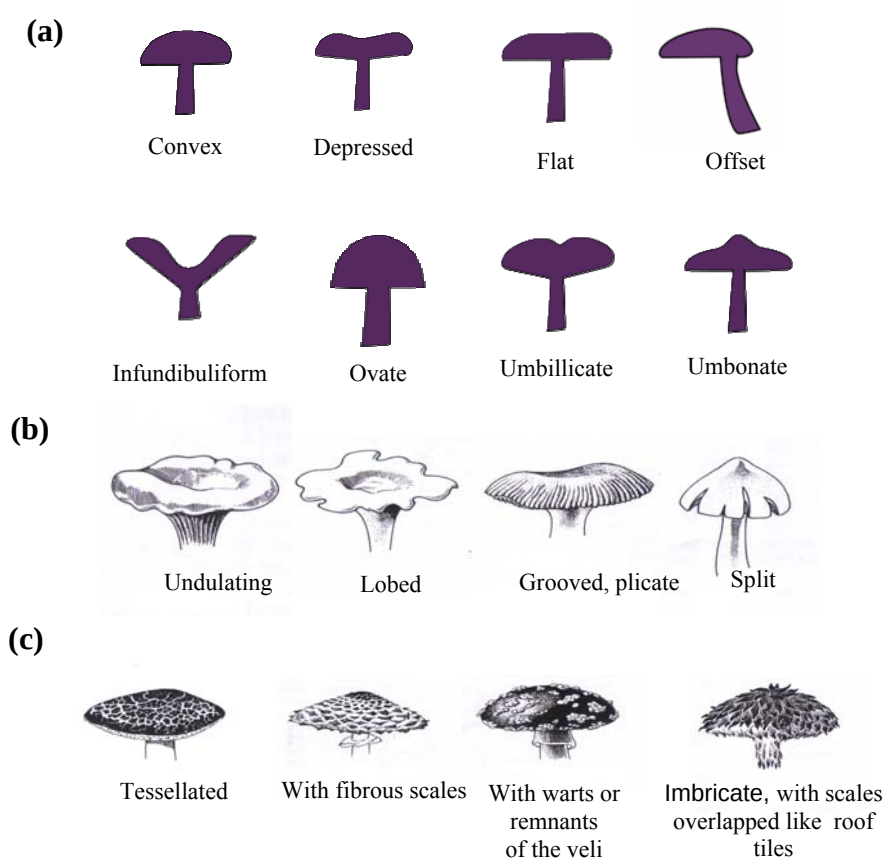


Figure 2.3 Various shapes of caps (a), cap edge (b) and cap surface (c).

Source: Svrček (2000)

A stalk (stipe) is feature supporting the cap of a mushroom. It is composed of sterile hyphal tissue, which has remains of a partial veil (annulus or ring) or universal veil (volva). It has general size and shape (Figure 2.4). Textures of the stalk are fibrous, brittle, chalky, leathery, or firm.

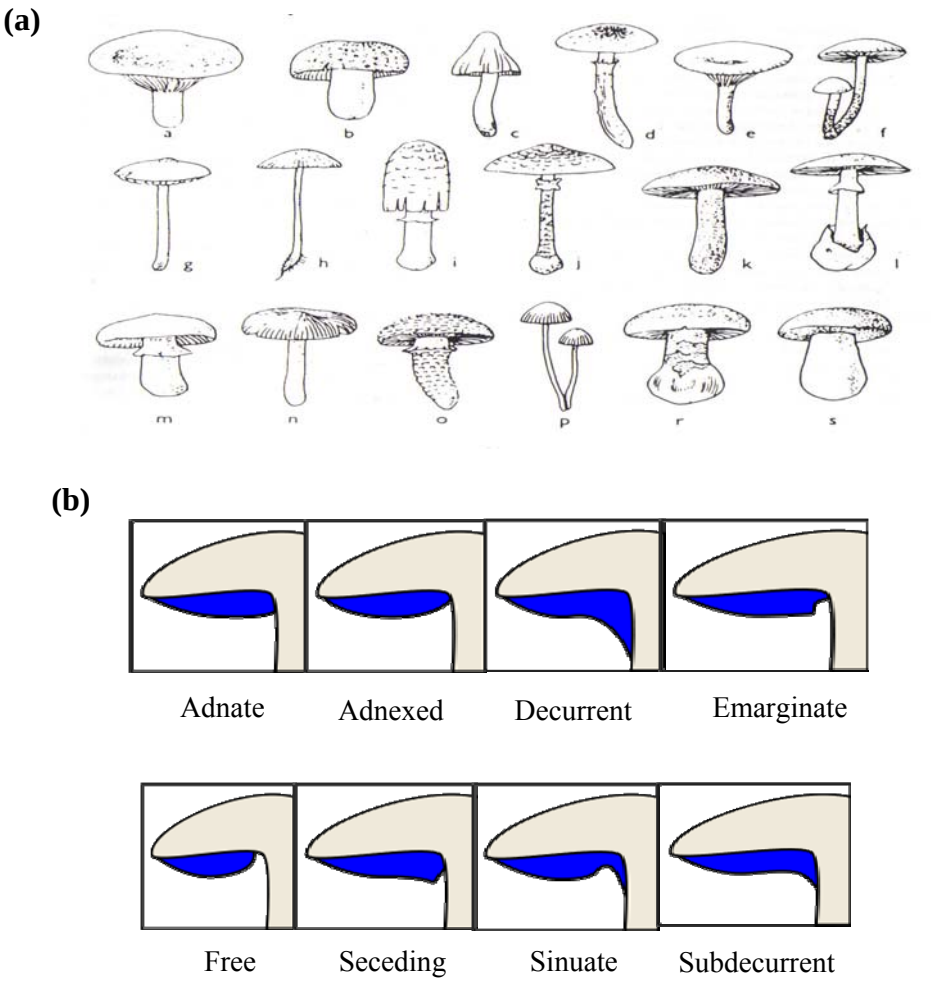


Figure 2.4 Various types of stalks (a) and the attachment of gill to the stalk (b). Source: Svrček (2000)

Spores of fungi are vital for the dispersal of the species. The size of spore is on average between 5 and 15 mu (thousandth of a millimeter). The form, color and structure are varying from species to species (Figure 2.5).

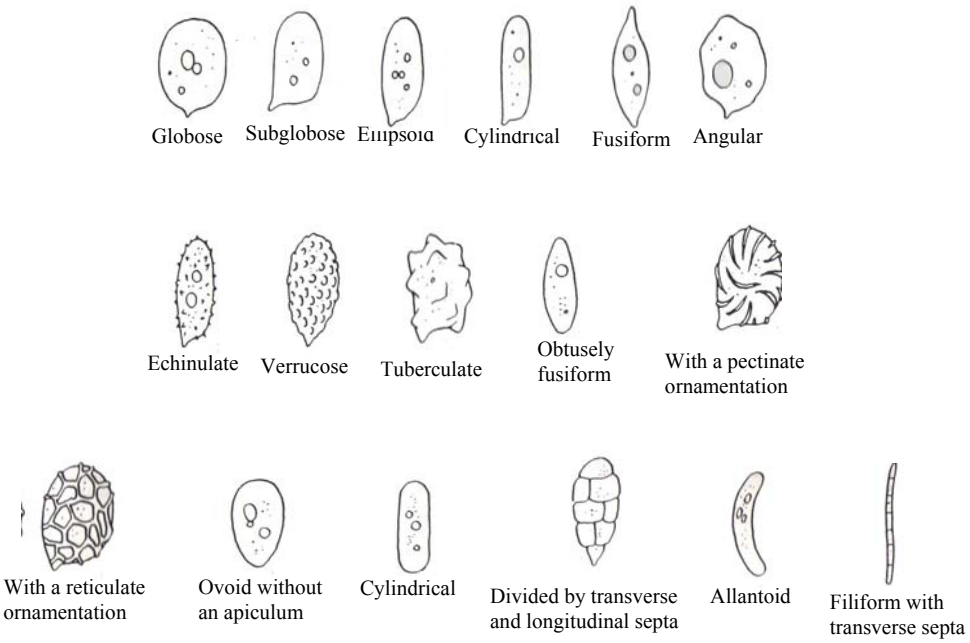


Figure 2.5 Various types of spores and ornamentation.

Source: Svrček (2000)

A hymenophore refers to the hymenium-bearing structure of a mushroom fruit body. Hymenophores can be smooth surfaces, lamellae, folds, tubes, or teeth, on the underside of the cap (Figure 2.6).

Figure 2.6 Various shapes of hymenophores.

Source: Source: Svrček (2000)

2.3 Mushrooms as a potential source of lectins

In mushrooms, lectins have been found to localize in caps, stipes and mycelia. The variation in lectin content occurs depending on age of the carpophore, time and place of harvest (Han *et al*., 2005). The great diversity of mushrooms has been reported in Thailand. These provide a high probability for lectin accumulation and exploitation. At least 90 genera of mushrooms have been reported to be found in National Forests of Thailand including Doi Inthanon National Forest, Doi Suthep National Forest, Khao Yai National Park (Walting, 1998), Nong-rawieng Plant Genetics Forest (Rodtong *et al*., 1998), Khao Kicha Koot National Park and Khao Soi Dao Wildlife Sanctuary (Klingesorn *et al*., 1998). The common families found are Agaricaceae, Coriolaceae, Ganodermataceae, Geastraceae, Hygrophoraceae, Hymenochaetaceae, Lycoperdaceae, Polyporaceae, Russulaceae and Tricholomataceae. The high incidence of lectin accumulations has been observed in mushroom specimens belonging to families; Agaricaceae, Cantharellaceae, Pleurotaceae and Tricholomataceae (Chumkhunthod, 2004). Lectins have been isolated from several species of edible mushrooms, including *Agaricus bisporus*, *Flammulina velutipes*, *Ganoderma lucidum*, *Grifola frondosa*, *Hericium erinaceum* and *Pleurotus ostreatus* (Ngai and Ng, 2004). *Volvariella volvacea* known as straw mushroom or paddy straw mushroom, and belonging to the family Pluteaceae of the Basidiomycetes, is a common edible mushroom of the tropics and subtropics. It can grow at relatively high temperature, i.e., vegetative growth at about 32 to 34°C. It is a fast-growing mushroom; the time required from spawning to harvesting is only about 8 to 10 days. It is widely cultivated in Thailand, and has been reported to accumulate lectins in its fruiting bodies (Wang *et al*., 1998).

2.4 Mushroom lectins

Mushroom lectins exhibit a diversity of chemical characteristics. They are a heterogeneous assembly of proteins with a wide range of molecular weights, subunit numbers, carbohydrate contents, sugar-specificities, amino acid sequences and structures (Lis and Sharon, 1998).

2.4.1 Chemical characteristics and properties of mushroom lectins

A) Molecular weights and subunits

Mushroom lectins display a range of molecular weights and the number of subunits. Their molecular weights can vary over the range from 12 to 190 kDa (Wang *et al*., 1998). Most mushroom lectins display mono-, di-, tri- or tetra-meric that may or may not be identical (Lis and Sharon, 1998). Some information of lectin properties isolated from mushrooms was summarized in Table 2.1.

B) Carbohydrate specificity

Traditionally, the specificities of lectins have been defined base on the simplest monosaccharides to inhibit hemagglutination or bind directly to the protein. Lectins are classified into five groups, according to the monosaccharide for which they exhibit the highest affinity: mannose, galactose/*N*-acetylgalactosamine, *N*-acetylglucosamine, fucose and *N*-acetylneuraminic acid (Figure 2.7). Relevant for the biological activities of lectins is the fact that of the numerous monosaccharides found in nature, only those listed above are typical constituents of surfaces of eukaryotic cells. The affinity of the lectins for monosaccharides is usually weak, with association constants in the millimolar (mM) range (Lis and Sharon, 1998). However, hemagglutinating activity of mushroom lectins from *Volvariella volvacea* (Lin *et al*.,

1984), *Agaricus blazei* (Kawagishi *et al*., 1988), *Flammulina velutipes* (Tsuda *et al*., 1997) and *Lyophyllum shimeiji* (Ng *et al*., 2002), were inhibited by glycoproteins but not by simple sugars.

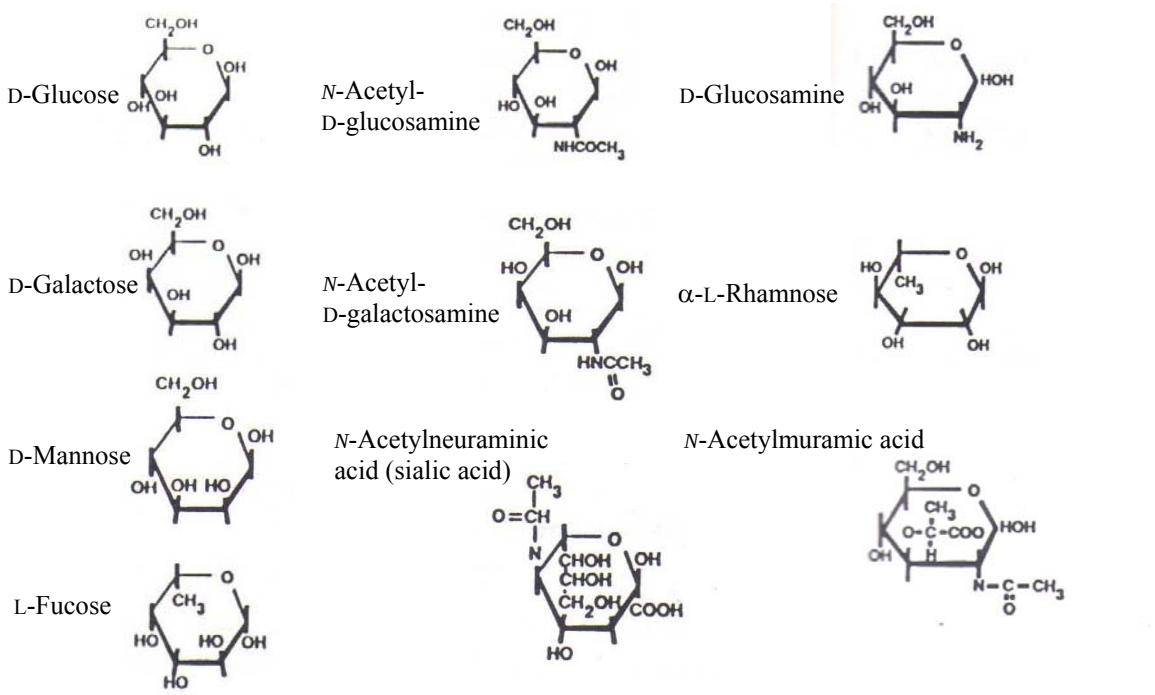


Figure 2.7 Common structure known to complex lectin-reactive carbohydrate structures.

Source: Sharon and Lis (1989)

C) Carbohydrate contents

The carbohydrate contents of mushroom lectins are ranging from 0 to 18% (Table 2.1) (Wang *et al*., 1998). No carbohydrate content has been found in some mushroom lectins of *Aleuria aurantia* (Kochibe and Furukawa, 1980), *Flammulina velutipes* (Yathogo *et al*., 1988), *Coprinus cinereus* (Cooper *et al*., 1997) and *Agrocybe aegerita* (Sun *et al*., 2003).

D) Amino acid sequences

Sequence similarity with known lectins provides a novel guideline for the detection and identification of new ones. Amino acid sequences of a lectin ultimately determine their overall three dimension structures and binding sites of the lectins. The comparision of amino acid sequences can be used to group the lectins (Figure 2.8).

E) Amino acid compositions of lectins

Several mushroom lectins have been analyzed for amino acid compositions. The amino acid composition of *Agrocybe aegerita* lectin showed a high contents of hydroxylic and acidic amino acids, low contents of phenylalanine, isoleucine and histidine residues, and traces of cysteine (Sun *et al*., 2003). Kochibe and Matta (1989) reported that the total amount of basic amino acids of *Psathyrella veilutina* lectin (PVL) was 49 residues/polypeptide molecule, while the acidic amino acids were 74 residues. The amino acid composition of *Agrocybe cylindracea* lectin was apparently different from *Agaricus campestris* lectin and *Pleurotus cornucopiae* lectin in the content of serine and basic amino acids (Yagi *et al*., 1997).

Table 2.1 Sources and some properties of lectins isolated from mushrooms.

Molecular structure	Lectin-producing mushroom	Abbreviation	MW (kDa)	Carbohydrate content (%)	Sugar specificity	Reference
Monomeric						
	Auricularia polytricha	-	23	3.5	Lactose, Galactose	Yagi and tadera (1988)
	Boletus satanas	-	63	-	Galactose	Kretz *et al*. (1989)
	Coprinus cinereus	-	16.4	0	Galactose	Cooper *et al*. (1997)
	Ganoderma capense	-	18	-	Galactose	Patrick *et al*. (2003)
	Peziza sylvestris	PSL	20	-	Arabinose	Wang and Ng (2005)
	Pleurotus eous	PEL	16	9	-	Mahajan *et al*. (2002)
	Psathyrella lacrymabunda	PAL	40	0.5	GalNAc	Kochibe and Matta (1989)
	Psathyrella velutina	PVL	40	0.5-0.7	GalNAc	Kochibe and Matta (1989)
	Xerocomus chrysenteron	XCL	17	3.5	-	Sychrova *et al*. (1985)
Dimeric						
	Agrocybe aegerita	AAL	32	0	Lactose	Sun *et al*. (2003)
	Agrocybe cylindracea	ACL	31.5	-	Lactose	Liu *et al*. (2007)
	Aleuria aurantia	AAL	72	0	Fucose	Fukumori *et al*. (1989)
	Armillaria luteo-virens	ALL	29.4	-	Inulin	Feng *et al*. (2006)
	Boletus edulis	-	32.5	-	Melibiose, Xylose	Zheng *et al*. (2007)
	Flammulina velutipes	FVA	20	0	-	Yathogo *et al*. (1988)
	Hericium erinaceum	HEL	54	1.5	-	Kawagishi *et al*. (1994)
	Ischnoderma resinosum	IRA	32	4	Lactulose, Galactose	Kawagishi and Mizuno (1988)

GalNAc: *N*-acetyl-D-galactosamine

Table 2.1 (Continued) Sources and some properties of lectins isolated from mushrooms.

Molecular structure	Lectin-producing mushroom	Abbreviation	MW (kDa)	Carbohydrate content (%)	Sugar specificity	Reference
Dimeric						
	Pleurotus citrinopileatus	-	32.4	-	Maltose, inulin	Li *et al*. (2008)
	Pleurotus ostreatus	POL	72	-	GalNAc	Wang *et al.* (2000)
	Polyporus adusta	-	24	-	Turanose	Wang *et al*. (2003)
	Schizophyllum commune	SCL	64	-	GalNAc, Lactose	Chumkhunthod *et al*. (2006); Han *et al*. (2005)
	Xerocomus spadiceus	-	32.2	-	Inulin	Liu *et al*. (2004)
	Xylaria hypoxylon	XHL	28.8	-	Inulin, Xylose	Liu *et al*. (2006)
Trimeric						
	Fomes fomentarius	-	60	25	GalNAc, Raffinose	Horejsi and Kocourek (1978)
Tetrameric						
	Agaricus bisporus	ABA I-IV	64	1.8-6.4	βGal(1,3) GalNAc	Sueyoshi *et al*. (1985)
	Agaricus blazei	-	64	11	-	Kawagishi *et al*. (1988)
	Agaricus campestris	-	64	4	-	Sage and Connett (1969)
	Agaricus edulis I	-	64	18	-	Eifler and Ziska (1980)
	Lactarius lignyotus	-	100	4	-	Sychrova *et al*. (1985)
	Phallus impudicus	-	75	13.9	-	Entlicher *et al*. (1985)
Hexameric						
	Ganoderma lucidum	-	114	9.3	-	Thakur *et al*. (2007)

GalNAc: *N*-acetyl-D-galactosamine

Figure 2.8 Comparison of amino acid sequences of mushroom lectins; ABL, *Agaricus bisporus*; PIL, *Paxillus involutus*; PCL, *Pleurotus cornucopiae*; XCL, *Xerocomus chrysenteron*. The residues conserved in all the members of the group are displayed on black background.

Source: Carrizo *et al*. (2005) ; Birck *et al*. (2004)

2.4.2 Physical properties of mushroom lectins

A) Temperature stability

Lectins from certain mushroom species exhibit their stability at certain temperature (Table 2.2). For example, *Ganoderma capense* lectin manifested remarkable thermostability. Complete retention of this lectin activity was observed after exposure of the lectin to 100°C for 1 h. Its temperature stability is more pronounced than what has been previously reported for other mushroom lectins such as lectins from *Agaricus* species, *Tricholoma mongolicum* and *Volvariella volvacea* (Wang *et al*., 1998). Whereas, lectins from *Hygrophorus hypothejus* (Veau *et al*., 1999), *Pleurotus ostreatus* (Wang *et al*., 2000) and *Boletus edulis* (Zheng *et al*., 2007), were much less temperature stability.

B) pH stability

Mushroom lectins could retain their activity in either acidic or basidic conditions (Table 2.2). The hemagglutinating activity *of Agrocybe aegerita* lectin was almost completely retained even after being exposured at pH 2.42-12.22. It was similar with *Vovariella volvacea* lectin, which was fully active at pH 2-11 (Lin *et al*., 1984).

Table 2.2 Temperature stability and pH stability of some mushroom lectins.

Lectins from mushroom species	Stability		Reference
	Temperature (°C)	pH	
Agaricus campestris	80	4.0-10.0	Sage and Vazquez *et al*. (1967)
Agrocybe aegerita	50	2.4-12.2	Sun *et al*. (2003)
Agrocybe cylindracea	60	6.0-9.1	Liu *et al*. (2007)
Armillaria luteo-viren	70	-	Feng *et al*. (2006)
Boletus edulis	40	-	Zheng *et al*. (2007)
Cordyceps militaris	50	6.0-9.1	Jung *et al*. (2007)
Flammulina velutipes	50	4.0-11.0	Liu *et al*. (2006)
Ganoderma carpense	100	-	Ngai and Ng (2004)
Ganoderma lucidum	50	5.0-9.0	Thakur *et al*. (2007)
Hericium erinaceum	70	5.0-10.5	Kawagishi (1994)
Hygrophorus hypothejus	40	4.0-10.0	Veau *et al*. (1999)
Mycoleptodonoides aitchisonii	45	4.0-9.0	Kawagishi *et al*. (2001)
Pleurotus citrinopileatus	60	-	Li *et al*. (2008)
Pleurotus ostreatus	30	-	Wang *et al*. (2000)
Schizophyllum commune	55	3.0-10.0	Chumkhunthod *et al*. (2006)
Tricholoma mongolicum	80	-	Wang *et al*. (1995)

-: Not determined

2.4.3 Biological properties of mushroom lectins

A) Agglutination reaction

Each lectin molecule contains typically two or more carbohydrate-combining sites. Therefore, they can react with cells or molecules, for example erythrocytes, polysaccharide or glycoprotein molecules in solution, by combining to the sugars on their surfaces, and also cause cross-linking the cells and their subsequent precipitation, a phenomenon referred to as cell agglutination. Both

agglutination and precipitation reactions of lectins are inhibited by the sugar ligands for which the lectins are specific. The erythrocyte agglutinating, or hemagglutinating, activity of lectins is a major attribute of these proteins, and is used routinely for their detection and characterization (Lis and Sharon, 1998).

B) Antiproliferative activity

A few lectins are being investigated for their use in cancer research and therapy. Preliminary findings suggest that some lectins but not all can detect alterations of malignant cells as well as reduce cancer cell tumorigenicity and thus may be helpful for prognosis of the immune status of patients (Gabius, 1987). For example, *Grifola frondosa* lectin is cytotoxic to HeLa cells (Kawagishi *et al*., 1990), *Agrocybe aegerita* lectin showed strong inhibition of human cervical carcinoma (HeLa), human colonic adenocarcinoma (SW480), human stomach cancer (SGC-7901), human gastric cancer (MGC80-3 and BGC-823) and human promyelocytic leukaemia (HL-60) cells (Zhao *et al*., 2003). *Tricholoma mongolicum* lectin (Wang *et al*., 1997), *Pleurotus ostreatus* (Wang *et al*., 2000), *Vovariella volvacea* (Lin *et al*., 1984) and *Pleurotus citrinopileatus* (Li *et al*., 2008) could inhibit mouse sarcoma 180 cells. Lectin from *Inocybe umbrinella* (Zhao *et al*., 2009) and *Pholiota adipose* (Zhang *et al*., 2009) showed antiproliferative activity toward hepatoma HepG2 cells and breast cancer MCF-7 cells.

C) Mitogenic activity toward lymphocytes

A limited number of lectins, mostly from plants possess the unique ability to induce quiescent lymphocytes to grow and divide, a phenomenon known as

mitogenic stimulation (Kilpatrick, 1998). There are a few reports of mushroom lectins with mitogenic activity. Lectins from mushrooms *Boletus satanas* Lenz (Licastro *et al*., 1993), *Lentinus edodes* (Jeune *et al*., 1990), *Ganoderma lucidum* (Kawagishi *et al*., 1997), *Agrocybe cylindracea* (Wang *et al*., 2002), *Ganoderma capense* (Patrick *et al*., 2003), *Volvareilla volvacea* (Ho *et al*., 2004), *Schizophyllum commune* (Han *et al*., 2005) and *Boletus edulis* (Zheng *et al*., 2007) exhibit potent mitogenic activity toward mouse lymphocytes, while other mushroom lectins from *Lactarius deliciosus* (Guillot *et al*., 1991), *Tricholoma mongolicum* (Wang *et al*., 1995) and *Laetiporus sulfureus* (Konska *et al*., 1994) are non-mitogenic.

D) Antiviral activity

Antiviral activities of some mushroom lectins have been reported. *Agrocybe aegerita* lectin showed inhibition activity to infection of tobacco mosaic virus (TMV) on *Nicotiana glutinosa* leaves (Sun *et al*., 2003). Some mushroom lectins displayed inhibition activity toward HIV-1 reverse transcriptase such as *Pleurotus ostreatus* (Wang *et al*., 2000), *Agaricus bisporus* (Wang *et al*., 2001), *Schizophyllum commune* (Han *et al*., 2005), *Boletus edulis* (Zheng *et al*., 2007), *Pleurotus citrinopileatus* (Li *et al*., 2008), *Inocybe umbrinella* (Zhao *et al*., 2009) and *Pholiota adipose* (Zhang *et al*., 2009).

E) Antifungal activity

Antifungal lectins have rarely been reported. Crude extract of *Schizophyllum commune* lectin showed some antifungal activity against *Aspergillus*

niger ATCC 6275, whereas the crude extract from *Stereum* sp. showed its inhibitory effect against yeast *Saccharomyces cerevisiae* ATCC 26108 (Chumkhunthod, 2004).

F) Antibacterial activity

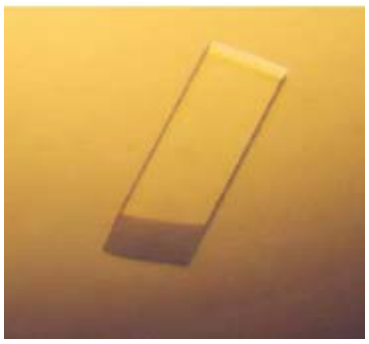
Lectins from several mushroom species are active against Gram-positive bacteria. Crude extracts of mushroom lectin from *Microporus* sp. showed considerable activity against *Bacillus cereus* ATCC 11778, while *Termitomyces microcarpus* (Berk.et Br) Heim and *Stereum* sp. had inhibitory activity with the Gram-positive bacterium, *Staphylococcus aureus* ATTCC 29213 (Chumkhunthod, 2004).

2.5 Crystal structures of mushroom lectins

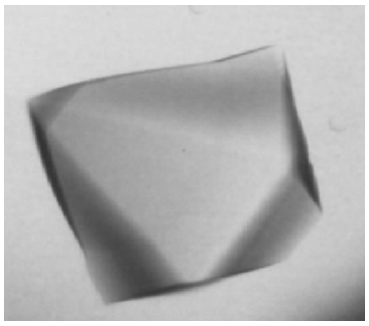
Lectins are employed in a wide variety of immunological and biochemical fields. They can be regarded as model systems for studying the molecular basis of protein carbohydrate interactions (Lis and Sharon, 1998). Due to biological roles of lectins, the interest in fungal lectins is increasing. Some mushroom lectins including *Pleurotus ostreatus* (Chattopadhyay *et al*., 1999), *Laetiporus sulphureus* (Mancheńo *et al*., 2004), *Agaricus bisporus* (Carrizo *et al*., 2004), *Marasmius oreades* (Grahn *et* al., 2004) and *Agrocybe aegerita* (Yang *et al*., 2005), have been successfully crystallized. Crystal of *Pleurotus ostreatus* lectin showed hexagonal bipyramid in shape, whereas the crystals of *Agrocybe aegerita* lectin showed columnar in shape (Figure 2.9).

X-ray crystal structures of very few mushroom lectins have been published (Figure 2.10). *Aleuria aurantia* fucose-binding lectin (AAL) was found to be a six-

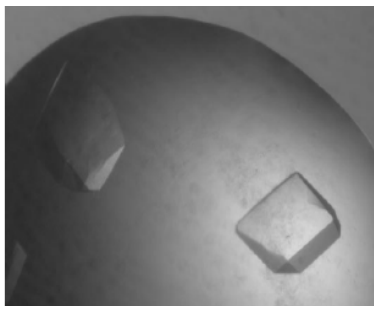
bladed β-propeller (Wimmerova *et al*., 2003). Lectin purified from *Flammulina velutipes* (Fve) displays structural similarity to human fibronectin (Paaventham *et al*., 2003). Whereas XCL, the lectin from *Xerocomus chrysenteron* (Birck *et al*., 2004) and ABL, the lectin from *Agaricus bisporus* (Carrizo *et al*., 2004), both resemble actinoporins, a family of pore-forming toxins. CGL2 the lectin from *Coprineus cinerea* (Walser *et al*., 2004) and ACG the lectin from *Agrocybe cylindracea* (Ban *et al*., 2005) belong to the galectin family. Recently, LSL, the lectin from of *Laetiporus sulphureus* (Mancheño *et al*., 2005) and PVL, the lectin from *Psathyrella velutina* (Cioci *et al*., 2006), have been reported.



Agrocybe aegerita lectin
Source: Yang *et al*. (2005)



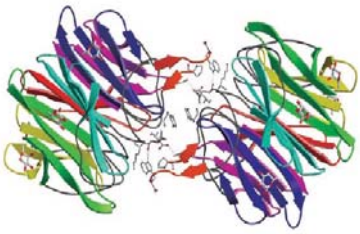
Pleurotus ostreatus lectin
Source: Chattopadhyay *et al*. (1999)



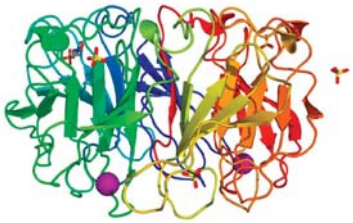
Agaricus bisporus lectin (ABL)
Source: Carrizo *et al*. (2004)

Marasmius oreades lectin (MOA)
Source: Grahn *et al*. (2004)

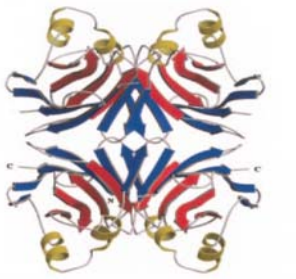
Figure 2.9 Crystal appearances of some mushroom lectins.



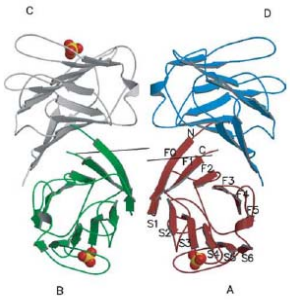
Aleuria aurantia lectin (AAL)
Source: Wimmerova *et al*. (2003)



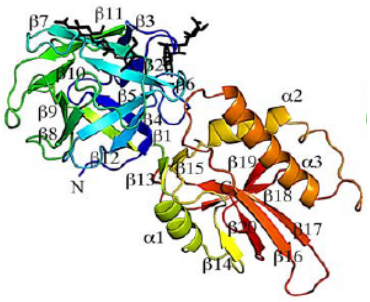
Psathyrella velutina lectin (PVL)
Source: Cioci *et al*. (2006)



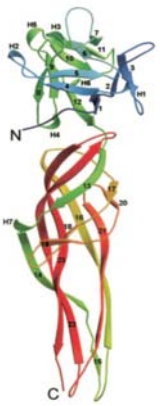
Agaricus bisporus lectin (ABL)
Source: Carrizo *et al*. (2004)



Agrocybe cylindracea lectin (ACG)
Source: Ban *et al*. (2005)



Marasmius oreades lectin (MOA)
Source: Grahn *et al*. (2007)



Laetiporus sulphureus lectin (LSL)
Source: Mancheño *et al*. (2005)

Figure 2.10 Ribbon diagrams of mushroom lectins.

2.6 Potential applications of lectins

Lectins are widely employed in pharmaceuticals, medicine, scientific research and agriculture because of their carbohydrate recognition which are base on precipitation and agglutination reactions (Lis and Sharon, 1998). Several of them have been commercially utilized (Wang *et al*., 1998), and can be regarded as model systems for studying the molecular basis of protein carbohydrate interactions (Lis and Sharon, 1998).

2.6.1 Pharmaceuticals

A) Mitogenic stimulation

Some lectins possess their unique ability to induce quiescent lymphocytes to grow and divide. Most mitogenic lectins stimulate only T-lymphocytes (T-cells), and are inactive on B-cells, such as concanavalin A and phytohemagglutinin (PHA). The mitogenic lectins are polyclonal activators, in that they activate lymphocytes irrespective of their antigenic specificity. Mitogenic stimulation by lectins provides an easy and simple means to assess the immunocompetence of patients suffering from a diversity of diseases, including AIDS and to monitor the effects of various immunosuppressive and immunotherapeutic manipulations (Lis and Sharon, 1998).

B) Cancer diagnosis

Lectins can be potentially used in cancer treatment strategies due to the fact that lectins present on the surface of tumor cells are capable of binding exogenous carbohydrate-containing molecules and internalize them by endocytosis. Lectins can

suppress growth of tumor cells in agarose, and inhibit lung colonization *in vivo*. A plant lectin, wheat germ agglutinin (WGA), is found to induce lectin-dependent macrophage-mediated cytotoxicity against human bladder cancer (T-24) cells and it found to enhance the cell killing ability of murine peritoneal macrphages *in vivo* (Ogawara *et al*., 1987).

C) Diabetes treatment

Lectins may be used in other interesting ways. In an automatic insulin dispensary device based on concanavalin A, the hormone has been modified by covalent attachment of glucose so that it could be bound by the immobilized concanavalin A in the device (Makino *et al*., 1991). When the glucose level in circulation rose, the modified insulin was dislodged from the immobilized concanavalin A, resulting in a rise in the level of insulin in plasma.

2.6.2 Medicine

A) Bone marrow transplantation

Some lectins have been used in organ transplantation. Soybean agglutinin (SBA) is routinely employed for the removal from bone marrow of immunologically non-identical donors of T-cells responsible for the lethal graft-versus-host disease, so that it can be safely transplanted into children born with severe combined immune deficiency (SCID) (Nagler, 1999). In another approach, T cell depletion of donor bone marrow has been achieved by treatment with an immunotoxin consisting of ricin and antibodies to T-lymphocytes. A ricin-linked anti-CD45 antibody has been recently shown to kill $CD4^+$ T-lymphocytes latently infected with

human immunodeficiency virus (HIV) in the blood of HIV-positive persons (Saavedra-Lozano *et al*., 2002).

B) Blood group typing

Lectins are used for distinguishing between erythrocytes of different blood types. The *Dolichos biflorus* lectin (DBL) is used to distinguish between blood type A_1 and A_2 subgroups (Lis and Sharon, 1998). The *Lotus tetragonolobus* lectin and *Ulex europaeus* (UEA-I) are served to identify blood type O cells, and *Vicia graminea* lectin is employed to differentiate between blood type M and N cells (Kilpatrick and Green, 1992). Moreover, the peanut lectin (PNA) which is specific for T-antigen Gla(β1-3)GalNAc, is used for the detection of polyagglutination in a condition certain bacterial and viral infections, in which human red blood cells become agglutinable by antibodies normally present in the sera of nearly all adults (Beck *et al*., 2000).

2.6.3 Research

A) Lectin derivatives preparation

For numerous purposes, lectin derivatives are required. For example, lectins derivatized with fluorescent dyes or biotin-avidin or radioactive compound, or enzymes or gold particles are employed as histochemical and cytochemical reagents with immunological techniques for detection of glycoconjugates in tissue sections, on cells and subcellular organelles, and in investigations of intracellular pathways of protein glycosylation (Rhodes *et al*., 1998). Immobilized lectins, covalently bound to Sepharose or other carriers, are indispensable for the purification and isolation by

affinity chromatography of glycoproteins, glycopeptides and oligosaccharides (Lis and Sharon, 1998).

B) Detection and assay of glycoconjugates

Lectins provide a simple means for detection and characterization of polysaccharides and glycoproteins in complex mixtures, for investigations of membrane structure, intracellular pathways of protein (Ewen *et al*., 1998). Lectin permits the examination of the connections between such neurons for localization of the membrane-associated glycoconjugates of discrete populations of neurons (Nakashima *et al*., 2000).

2.6.4 Agriculture

Biocontrol agents

Lectins may have their potential as bioinsecticides. Some plant lectins from snowdrop, pea, wheat, rice and soybean are toxic to insects (Atkinson *et al*., 2003). For instance, a lectin from snowdrop, *Galanthus nivalis* agglutinin (GNA), is toxic to several insect pests in the orders Homoptera, Coleoptera and Lepidoptera (Tinjuangjun *et al*., 2002). Concanavalin A suppresses *Meloidogyne incognita* when used as a soil amendment in a tomato crop (Marban-Mendoza *et al*., 1987). Recently, the lectin from mushroom *Agrocybe aegerita* displays antiphytoviral activity against tobacco mosaic virus (TMV) on *Nicotiana glutinos*a leaves (Sun *et al*., 2003). Thus, lectins are possible for the application in crop protection against insect, nematode and phytopathogenic viruses.

CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals, reagents and media

Chemicals, reagents and media were used in each step as follows:

3.1.1 Morphological characterization of straw mushroom fruiting bodies

Reagent used for microscopic study of straw mushroom cultivated from different locations was lactophenol (Appendix A7).

3.1.2 Determination of lectins accumulated in straw mushroom specimens

Reagent used for extraction of crude lectin extracts was 10 mM phosphate buffer saline (PBS, pH 7.4) containing 2 mM sodium bisulphite (Appendix A1.1). 2% suspension of rabbit red blood cells (RBC) in PBS, pH 7.4 was used to determine the lectins accumulated in straw mushroom specimens.

3.1.3 Determination of biological properties of straw mushroom lectin extracts

3.1.3.1 Antimicrobial activity test

Culture media used for determination of antimicrobial activity test were nutrient broth, Mueller Hinton agar (MHA), malt extract broth and malt extract agar (Appendix A8).

3.1.3.2 Cytotoxicity test against human cancer cells

The medium used for culturing the human cancer and normal cells was minimum essential medium (MEM) containing 1% antibiotic-antimycotic and 10% fetal bovine serum (FBS). Chemicals and reagents used for determination of cytotoxicity were PBS, pH 7.4 to wash the cells; trypsin-EDTA solution to trypsinize the cells; MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) solution to determine cytotoxicity; dimethyl sulfoxide (DMSO) to dissolve a purple formazan product. All products were purchased from GIBCO® (Invitrogen, U.S.A.).

3.1.4 Purification of the selected straw mushroom lectin

Reagents used for lectin purification were 10 mM PBS, pH 7.4, containing 1 mM benzamidine, 0.1% 2-mercaptoethanol and 1.5% (w/v) polyvinylpyrrolidone (PVPP) (Appendix A1.2) to extract crude lectin; 10 mM Tris-HCl, pH 8.0 to dissolve proteins; solid ammonium sulfate to precipitate proteins (Appendix B); Bradford dye to estimate of total protein in straw mushroom lectin extracts (Appendixes A5 and B).

Reagents used for determination of molecular weights of purified straw mushroom lectins, were sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), 12.5% polyacrylamide gel (Appendix A3.1), low molecular weight protein marker (Amersham-Pharmacia Biotech, Uppsala, Sweden), SDS-gel loading buffer (Appendix A3.4), staining solution with coomassie brilliant blue R-250 (Appendix A3.5), and destaining solution with coomassie (Appendix A3.6).

3.1.5 Characterization of the purified straw mushroom lectin

3.1.5.1 Glycoprotein property

Reagent used for determination of carbohydrate of the lectins was periodic acid Schiff (PAS) staining (Appendix A4). Ribonuclease B (RNase B), product of Sigma-aldrich (St. Louis, U.S.A.), was used as positive control

3.1.5.2 Sugar specificity

Chemicals and reagents used for studying sugar specificity of the straw mushroom lectin were PBS, pH 7.4, 2% suspension of rabbit red blood cells and different types of sugars; *N*-acetyl-D-galactosamine (GalNAc), 2-acetamido-2-deoxy-D-glucopyranose, D-adonitol, amygdalin, L-arabinose, cellobiose, esculin, fructose, fucose, D-galactose, D-glucose, inositol, inulin, lactose, lactitol, maltose, mannitol, D-mannose, D-melezitose, melibiose, methyl-α-D-galactopyranoside, methyl-β-D-galactopyranoside, 4-nitrophynyl-α-D-galactopyranoside, 4-nitrophynyl-β-D-galactopyranoside, raffinose, rhamnose, ribose, salicin, sorbitol, trehalose, D-turanose and D-xylose. All products were purchased from Merck (Merck & Co., Inc., U.S.A).

3.1.6 Crystallization of the selected straw mushroom lectin

Reagents used for crystallization screening were Crystal Screen HR2-110 and HR2-112 (Hampton Research, California, U.S.A).

3.1.7 Genetic characterization of the selected straw mushroom specimen

Reagents used for genomic DNA extraction were lysis buffer (Appendix A6.1); phenol, chloroform, isoamyl alcohol (Merck) to purify genomic DNA;

isopropyl alcohol (Merck) to precipitate genomic DNA; 70% ethanol to wash genomic DNA pellet. Agarose (Promega, Promega Coporation, U.S.A.) was used to detect DNA by agarose gel electrophoresis.

Reagents used for Polymerase Chain Reaction (PCR) amplification were the 10X PCR buffer, dNTPs (dATP, dCTP, dGTP and dTTP) and *Taq* DNA polymerase (Promega). The oligonucleotide primers were ordered from the Science Pacific Company, Ltd., Thailand. The Gel/PCR DNA Fragments Extraction Kit (Geneaid, Geneaid Biotech, Ltd., Taiwan) was used in PCR purification. The BigDye Terminator Ready Reaction kit (Perkin Elmer, Applied Biosystems Inc., U.S.A.) was used for the nucleotide sequencing reaction.

3.2 Instrumentation

All instruments required for identification of straw mushroom specimens, determination of lectins accumulated in mushroom specimens, protein purification, and characterization, were located at the Instrument Buildings of the Center for Scientific and Technological Equipment, Suranaree University of Technology, Nakhon Ratchasima, Thailand. Instruments used for cytotoxicity assay, were located at the Natural Products Research Section, Research Division, National Cancer Institute, Bangkok, Thailand. For determination of isoelectric point (pI) and amino acid sequence of straw mushroom lectin, the instruments were located at the Genome institute, National Center for Genetic Engineering and Biotechnology, Pathumthani, Thailand. For lectins crystallization and X-ray diffraction, the experiment was conducted at Macromolecule Crystallography endstation of Synchrotron Light Research Institute (Public Organization) (SLRI) Nakhon Ratchasima, Thailand.

3.3 Collection and preservation of straw mushroom specimens

Fresh fruiting bodies of straw mushroom specimens cultivated from different locations in North-eastern Thailand were collected during May to July in 2008, from local markets in North-eastern Thailand. The mushroom specimens collection was performed according to the protocol described by Svrček (1996). Data concerning the mushroom, collection locality and date, were recorded. After returning to the laboratory, specimen morphology was characterized. The collected fruiting bodies were then dried in the hot air oven at 40°C for 6-12 h. The dried specimens were used for lectin extraction.

3.4 Morphological characteristics of straw mushroom fruiting bodies

The straw mushroom specimens cultivated from different locations were morphological characterized. Shape, size and color of their caps, stalks and spores were observed and recorded. Gills from the cap of each specimen were sectioned using a razor blade and examined under light microscope.

3.5 Determination of lectins accumulated in straw mushroom specimens

3.5.1 Extraction of crude lectins

Cap and stalk of mature fruiting bodies, as well as the whole fruiting body of straw mushroom specimens were separately crushed into powder using blender. Ten grams of the powder were extracted with 10 times (w/v) of 10 mM PBS, pH 7.4,

containing 2 mM sodium bisulphate, then left at 4°C for 2 h. The mixtures were filtrated through a double layer of cheesecloth to remove any large impurities, and centrifuged at 10,000×g for 30 min at 4°C. The supernatant obtained, was defined as crude extract, which was used for the detection of lectin accumulations using hemagglutination assay.

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3.5.2 Detection of lectins by hemagglutination assay

Hemagglutination has been performed as the simple method for all lectin determination. Accumulations of lectins in crude extracts of straw mushroom specimens were detected by hemagglutinating activity against rabbit red blood cells. In the assay, a serial two-fold dilution of crude lectin extracts solution in microtiter plate (130×85×15 mm) containing 8×12 wells with U-shaped bottom (50 µl) was mixed with 50 µl of a 2% suspension of rabbit red blood cells in PBS, pH 7.4, and incubated at 4°C. Hemagglutination results were recorded after 1 h when the negative result (no lectin was added) had fully precipitated. In contrast to positive result that agglutination occurred, when red blood cells formed a red carpet which covered the whole bottom of the microtiter plate well. The hemagglutination titer, defined as the reciprocal of the highest dilution exhibiting hemagglutination, was reckoned as one hemagglutination unit. Specific activity is the number of hemagglutination units/mg protein.

3.5.3 Estimation of total protein in straw mushroom lectin extracts

Total protein concentration in straw mushroom lectin extract was estimated by the method of Bradford *et al*. (1976), using bovine serum albumin

(BSA) as a standard protein (0-10 μg) (Appendix B). A diluted sample (100 μl) was mixed with 1 ml of Bradford dye reagent and 50 μl of 1 M sodium hydroxide (NaOH). The solution was mixed and allowed to stand at room temperature for 10 min. Absorbance at 595 nm was measured using the Lambda 40 UV-VIS spectrophotometer (Perkin Elmer Instruments, Shelton, CT, U.S.A.).

3.6 Determination of biological properties of straw mushroom lectin extracts

3.6.1 Extraction of crude lectins for testing biological properties

In order to clarify their biological properties, the purity of the straw mushroom extracts must be considered. Polyvinylpyrrolidone (PVPP) powder was added to the lectin extraction for absorbing polyphenolic substances. Lectin was extracted from dried fruiting bodies of straw mushroom powders. The powders were homogenized in a cold mortar and pestle with 10 times (w/v) of 10 mM PBS, pH 7.4, containing 1 mM benzamidine, 0.1% 2-mercaptoethanol and 1.5% (w/v) PVPP. The homogenate was stirred at 4°C overnight, then filtrated through a double layer of cheesecloth, and centrifuged at 10,000×g at 4°C for 30 min. The supernatant was obtained and consequently submitted to biological activity test

3.6.2 Antimicrobial activity test

Crude lectin extracts and the purified lectin were sterilized by filtration through a 0.45 μm membrane filter. Agar disc diffusion method was carried out according to Murray *et al*. (1999).

3.6.2.1 Antibacterial activity

Test organisms used for the antibacterial assay, *Bacillus cereus* ATCC 11778, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922 were carried out in petri plates (100×15 mm) containing 20 ml of Mueller Hinton agar (MHA) plates. 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 37°C. Then the overnight culture was diluted with PBS, pH 7.4 to yield a 0.5 McFarland suspension (approximately 10^8 CFU/ml). The prepared inoculum was then inoculated on Mueller Hinton agar (MHA) plate by three-dimension swab technique (streaking each plate in horizontal, vertical directions and around the edge with a sterile swab). The sterile blank paper discs (6 mm in diameter, Whatman, U.K.) were each impregnated with 20 µl of either crude lectin extracts or purified lectins, and placed on the inoculated agar, then incubated at 37°C for 24 h. Each test was carried out in triplicates with controls. The paper disc containing standard streptomycin (10 µg/20 µl), was used as the positive control.

3.6.2.2 Antifungal activity

For the antifungal activity assay, *Aspergillus niger* ATCC 6275, *Candida albican* ATCC 10231, *Penicillium funiculosum* ATCC 36839, *Saccharomyces cerevisiae* ATCC 9896 and *Saccharomyces cerevisiae* ATCC 18824, were grown in 2% (w/v) malt extract broth for 48 h at 30°C, then streaked on 2% (w/v) malt extract agar for the agar diffusion assay. The concentration of the fungal test organism was adjusted to 10^7 cells or spores/ml. The lectin extracts were applied as described in section 3.6.2.1. One hundred units of standard nystatin were used as

the positive control. In this test, the inhibitory zone diameter was detected after incubation for 48 h at 30°C. All tests were also performed in triplicates with controls.

3.6.3 Cytotoxicity test against human cancer cells

Crude lectin extracts and the purified lectin were tested against two types of human cancer cells: human epidermoid carcinoma (KB) and human cervical carcinoma (HeLa), and normal cells of African green monkey kidney epithelial (Vero) cells. These cells were maintained in minimum essential medium (MEM) supplemented with 1% antibiotic-antimycotic and 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO_2. Cell suspensions (90 µl/well) were prepared at a density of 3×10^4 cells/ml and seeded in 96 well flat bottom microtiter plates for 24 h before addition of the lectin samples. After discarding the culture medium and washing the cells with PBS, 10 µl of complete medium containing the lectin samples at various concentrations were then added and further incubation for 72 h in the same condition. At the end of the incubation, the medium was removed and the plate was washed with PBS. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) solution (100 µl/well) was then added following by further incubation for 3 h. Absorbance at 550 nm was measured using microtiter reader after incubation with dimethyl sulfoxide (100 µl/well) for 30 min. For the positive control experiment, cells were treated with 100 µl of complete medium containing 100 µM of adriamycin and the negative control experiment was performed using the same medium without drug. The 50% inhibitory concentration (IC_{50}) values which less than 30 µg/ml (crude extract) and less than 4 µg/ml (purified sample) were designated as cytotoxicity.

3.7 Selection of straw mushroom lectin according to their biological properties

Results from antibacterial, antifungal and cytotoxic activities of crude lectins extracts obtained from all straw mushroom specimens were compared. Only the mushroom specimen that showed promising potency was selected for further investigation of its lectin.

3.8 Purification of the selected straw mushroom lectin

3.8.1 Extraction of the selected straw mushroom lectin from fruiting bodies

Crude lectins were extracted from the selected straw mushroom specimen using the method as described by Chumkhunthod (2004). One hundred grams of ground fruiting bodies were homogenized with 10 times (w/v) of 10 mM phosphate buffer saline (PBS, pH 7.4) containing 1 mM benzamidine and 0.1% 2-mercaptoethanol. The homogenate was, then, added with 1.5% (w/v) polyvinylpyrrolidone (PVPP), and stirred at 4°C overnight. The homogenate was filtrated through double cheesecloth and centrifuged at 10,000×g for 30 min at 4°C. Supernatant was processed to lectin purification.

3.8.2 Preliminary study of purification methods for the selected straw mushroom lectin

For the preliminary study of straw mushroom, different types of affinity column; porcine stomach mucin (PSM)-Sepharose 4B, ion exchange chromatography

(DEAE-Sepharose and CM-Sepharose) were investigated. The suitable purification procedure achieved was used in the straw mushroom lectin purification.

3.8.3 Ammonium sulphate precipitation

A solid ammonium sulfate ($(NH_4)_2SO_4$) powder was added to supernatant containing lectin, to get 90% saturation (Appendix B). The mixture was stirred at 4°C overnight before centrifugation at 12,000×g for 30 min. The precipitate was collected, redissolved in a small volume of 10 mM Tris-HCl, pH 8.0, and dialyzed extensively against 10 mM Tris-HCl buffer, pH 7.3 before loading to ion exchange chromatography using DEAE-Sepharose column (GE Healthcare, Uppsala, Sweden).

3.8.4 Purification of the selected straw mushroom lectin by ion-exchange chromatography

The dialyzate of lectin was then applied on a DEAE-Sepharose column (1.5×11 cm; 9 ml bed volume) which had been pre-equilibrated with running buffer; 10 mM tris-HCl, pH 7.3, and exhaustively washed with the 3 bed volumns of the same buffer. Bound lectin was eluted with a stepwise gradient increasing concentration of sodium chloride (NaCl) from 0 to 0.5 M in the running buffer at a flow rate of 24 ml/h. Afterwards all fractions of 1 ml were collected using a fraction collector, model Frac-920 (GE Healthcare, U.S.A.). The 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 against rabbit erythrocytes was determined in the resulting fractions, and the active fractions were identified as purified lectin. The purified lectin fractions were

then pooled, dialyzed, concentrated down by Vivaspin concentrator (molecular weight cut-off 10 kDa) (GE Healthcare, Uppsala, Sweden), and stored in the refrigerator (4°C) for further investigation.

3.8.5 Purification of the partial purified lectin by gel filtration chromatography

The partial purified of straw mushroom lectin obtained from the DEAE-Sepharose column was further subjected to gel filtration chromatography using an ÄKTA purifier system (Amersham Biosciences, New Jersey, U.S.A.) on a Superdex 75 (GL 10/300) column (GE Healthcare). Gel filtration calibration kit low molecular weight (GE Healthcare) containing conalbumin (75 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), ribonuclease A (13.7 kDa), aprotinin (6.5 kDa) and blue dextran (2,000 kDa) was used to estimate native molecular weight of lectin. The running buffer was 10 mM Tris-HCl buffer, pH 8.0 containing 100 mM NaCl. A flow rate of 30 ml/h was maintained, and 0.5 ml fractions were collected and assayed for hemeagglutinating activity.

3.8.6 Dialysis of the purified lectin

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 semipermeable 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 4°C 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.

3.8.7 Concentration of protein

The concentration of protein in the solution had to be considerated. It should be in the range of 0.5 to 1 mg/ml or higher. Thus the volume of protein was decreased down by Vivaspin ultrafiltration spin columns (GE Healthcare, Uppsala, Sweden) with polyethersulfone 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.

3.9 Characterization of the purified straw mushroom lectin

The purified straw mushroom lectin was characterized as follows:

3.9.1 Chemical properties of the purified straw mushroom lectin

3.9.1.1 Molecular weight

The molecular weight of the purified lectin from straw mushroom was determined by both sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and gel filtration. The purified lectin was subjected to SDS–PAGE for molecular weight of lectin subunits using the method as described by Laemmli and

Favre (1970). SDS-polyacrylamide gel electrophoresis was performed by using a discontinuous system with a 12.5% acrylamide running gel and 5% stacking gel. After electrophoresis, the gel was stained with coomassie brilliant blue R-250 (Appendix A3.5) for 30 min, and then destained with a destaining solution (Appendix A3.6) until the background is clear. The size of protein bands were estimated by comparing with the low molecular weight protein marker (GE Healthcare, Uppsala, Sweden) comprising phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), bovine carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa) and bovine α-lactalbumin (14 kDa).

Gel filtration chromatography for determining the molecular weight of native lectins was carried out on a Superdex 75 (GL 10/300) column (GE Healthcare), which had been calibrated with gel filtration calibration kit low molecular weight protein markers (GE Healthcare) including conalbumin (75 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), ribonuclease A (13.7 kDa), aprotinin (6.5 kDa), and blue dextran (2,000 kDa).

3.9.1.2 Isoelectric point (pI)

The isoelectric point (pI) of the purified lectin was determined using 2D-electrophoresis according to principles and methods of 2D- electrophoresis from GE Healthcare Bio-Science UK Ltd. The first-dimension step, isoelectric focusing (IEF), separates proteins according to their isoelectric points (pI) using 7 cm IPG strip, pH range 3.0-10.0. After IEF, the second-dimension step, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), separates proteins according

to their molecular weights. Each spot on the resulting two-dimensional gel potentially corresponds to a single protein species in the sample.

3.9.1.3 Glycoprotein property

In order to determine glycoprotein property of the lectins, periodic acid Schiff (PAS) staining was investigated according to the method described by Fukuda and Kobata (1993). The modified PAS staining technique for glycoprotein was performed as follows: the electrophoresis gel from SDS-PAGE was incubated for 30 min in fixation solution (12.5% TCA) and following by soaking in periodic acid solution (Appendix A4.3) for 50 min. After rinsing the gel twice with distilled water, the gel was then placed in Schiff's reagent (fuchsin-sulphite solution) (Appendix A4.5) and incubated in the dark for 50 min. Finally, the gel was placed in 0.5% potassium meta-bisulphite solution (Appendix A4.4) and left for 10 min (3 times for 10 min each change of 30 ml per gel). The gel was rinsed in water until red band of carbohydrate appeared. No more staining intensity was usually seen after 2 h. Ribonuclease B, product of Sigma-aldrich (U.S.A.), was used as the positive control.

3.9.1.4 Amino acid sequence

For protein identification, the purified lectin band from the SDS-PAGE, was excised and in-gel digested with trypsin (sequencing grade, Promega, Madison, U.S.A.) using a standard protocol (Shevchenko *et al*., 1996). After overnight digestion at 37°C, the peptides were extracted and dried in a SpeedVac vacuum centrifuge. A small fraction of these tryptic peptides were analyzed with liquid chromatography-electrospray ionization mass spectrometry (LC/ESI/MS)

(Bruker nanoLC-HCTultra system, Bruker Daltonik GmbH, Bremen, Germany). MS/MS data of digested peptides were analyzed by MS/MS ion search in MASCOT search engine (Matrix Science Ltd., London), which used mass spectrometry data to identify proteins from primary sequence databases.

3.9.1.5 Sugar specificity

To investigate inhibition of lectin-induced hemagglutination by various sugars were performed in a manner analogous to the hemagglutination tests. The sugars tested included *N*-acetyl-D-galactosamine (GalNAc), 2-acetamido-2-deoxy-D-glucopyranose, D-adonitol, amygdalin, L-arabinose, cellobiose, esculin, fructose, fucose, D-galactose, D-glucose, inositol, inulin, lactose, lactitol, maltose, mannitol, D-mannose, D-melezitose, melibiose, methyl-α-D-galactopyranoside, methyl-β-D-galactopyranoside, 4-nitrophynyl-α-D-galactopyranoside, 4-nitrophynyl-β-D-galactopyranoside, raffinose, rhamnose, ribose, salicin, sorbitol, trehalose, D-turanose and D-xylose were investigated. Serial two-fold dilutions of sugar samples were prepared in PBS, pH 7.4. All of the dilutions were mixed with an equal volume (25 µl) of the purified lectin solution of the agglutinin with 16 (2^4) hemagglutination units. The mixture was allowed to incubate for 30 min at 4°C, and then mixed with 50 µl of a 2% rabbit red blood cells suspension. The minimum concentration of the sugar in the final reaction mixture that completely inhibited 16 hemagglutination units of the lectin preparation was calculated.

3.9.2 Physical properties of the purified straw mushroom lectin

Lectin solutions (1 mg/ml lectin solution in PBS, pH 7.4) of crude lectin and the purified lectin were investigated for testing the temperature and pH stabilities.

3.9.2.1 Temperature stability

For testing the temperature stability, lectin solutions were preincubated for 30 min at different temperatures of -20, 4, 30, 40, 50, 60, 70, 80, 90 and 100°C in a dry heat block. Then the heated lectin solutions were cooled rapidly on ice, centrifuged to remove any precipitate and assayed for hemagglutination in comparison with a control sample of lectin. The hemagglutination activity of each of the treated lectins was then determined as described above. Results were expressed by calculating the percentage of hemagglutination shown by the heated aliquots (titration value) compared with the control sample with representing 100%.

3.9.2.2 pH stability

The pH stability of the lectin was measured by incubating the lectin samples in the following buffers varying from pH 2.0-12.0 for 18 h at 4°C. 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 4.0-5.0), 50 mM phosphate buffer (pH 6.0-7.0), 50 mM Tris-HCl buffer (pH 8.0-8.5) and 50 mM glycine-NaOH buffer (pH 9.0-12.0). The residual hemagglutinating activity was assayed after dialysis the lectin samples against PBS, pH 7.4. Three replicates were done for each test. The control groups were treated with PBS, pH 7.4. Results were calculated by expressing the titration values of the lectin as percentages of the titration value of the control.

3.10 Preliminary study of the selected straw mushroom lectin structure

3.10.1 Crystallization of the selected straw mushroom lectin

In order to crystallize a protein, the purified protein undergoes slow precipitation from an aqueous solution. The macromolecular sample should be homogenous, as pure as practically possible (>95%), and free of amorphous and particulate material. The importance of protein crystallization is that it serves as the basis for X-ray crystallography, wherein a crystallized protein is used to determine the protein's three-dimensional structure via X-ray diffraction. In this study, the purified lectin was dissolved in 10 mM Tris-HCl, pH 8.0 for crystallization.

3.10.1.1 Determination of crystallization conditions using screening kit

The crystal screen was used for finding initial crystallization conditions. A highly effective approach to overcome the exhaustive search for suitable crystallization conditions was the use of a sparse matrix method of trial conditions that was biased and selected from known crystallization conditions for macromolecules. Initial crystallization screening was performed using the microbatch under oil technique with Crystal Screen HR2-110 and Crystal Screen HR2-112 (Hampton Research, California, U.S.A.). Each crystallization drop contained equal amounts (1 μl) of purified lectin (25 mg/ml) and reservoir with precipitant solution was incubated at 18°C. After setting up the screen, the lectin drops were immediately examined under a Stemi 2000-C, Stereo Microscope (Zeiss, Carl Zeiss MicroImaging, Inc., New York, U.S.A.). The lectin drops were observed daily for the first week and

then continued once a week there after. Results were recorded by indicating whether the lectin drop is clear, or contained precipitates and or crystals.

3.10.1.2 Optimization of crystal growth conditions

Based on the screen results, some parameters, particularly protein concentration, ammonium sulphate salt concentration and pH of crystallization solution, must be optimized for crystal growth. The hanging drop vapor diffusion method was used for further optimization of growing a crystal of lectin. Through optimization, the crystallization drops were formed by mixing equal volumes (1 μl) of lectin (5 mg/ml in 10 mM Tris-HCl, pH 8.0), and reservoir with precipitant solution and equilibrated over 1.0 ml of the respective precipitant in 24-well VDX^{TM} plate with sealant (Hampton Research, California, U.S.A.). To obtain better quality of protein crystals, both microseeding and macroseeding techniques could be further applied. Lectin concentration used were 1.5-5.0 mg/ml. Hanging drop technique was shown in Figure 3.1.

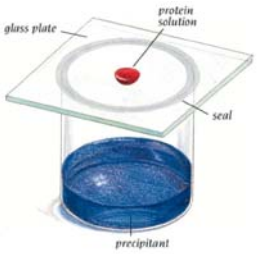

Figure 3.1 Diagram of hanging drop method. Reservoir solution (blue) usually contains buffer and precipitant. Protein solution (red) contains the same compounds, but in lower concentrations. The protein solution may also contain trace metals or ions necessary for precipitation of particular proteins.

Source: Creighton (1993).

3.10.2 X-ray diffraction of the selected straw mushroom lectin crystals

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 was mounted between an X-ray source and an X-ray detector. The crystal located in the path of a narrow beam of X-ray and diffracted the X-ray beam into many discrete beams, which produced distinct spots called reflections on the detector (Rhodes, 2000) (Figure 3.2).

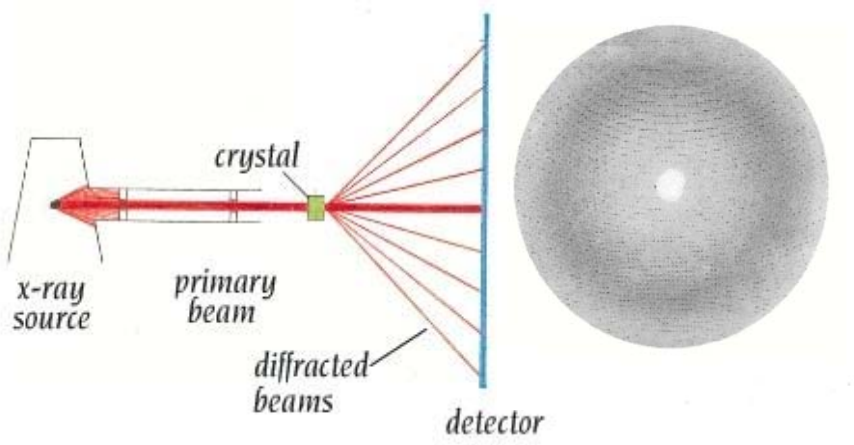

Figure 3.2 Crystallographic data collection.

Source: Thomas Creighton (1993).

Trial exposures of the lectin crystal were performed as X-ray diffraction experiments, which were carried out at 100 K using a Microstar™ rotating-anode X-ray generator at the Synchrotron Light Research Institute (Public Organization) (SLRI), Thailand, and a Mar165 charge-coupled device (CCD) detector on Macromolecule Crystallography endstation (Appendix E). Before data collection, the

mother liquor containing 15% glycerol was pipetted onto the crystal-containing drops and the crystals were transferred directly to liquid nitrogen. Then the crystals frozen were mounted in the beam and adjusted carefully on a goniometer in a stream of liquid nitrogen at 100 K. After the exposure, X-ray diffraction patterns were collected on the CCD detector.

3.11 Genetic characterization of the selected straw mushroom specimen

Analysis of the nucleotide sequence of 18S ribosomal RNA gene (18S rRNA gene) was used for genetic characterization. There were four major steps including extraction of genomic DNA, PCR amplification of 18S rRNA gene, sequencing of PCR amplification and analysis of 18S rRNA gene sequence.

3.11.1 Extraction of genomic DNA

Genomic DNA of the straw mushroom was extracted from fruiting body. The fruiting body was ground, added lysis buffer (25% SDS, 50 mM EDTA, 75 mM NaCl, 50 mM Tris-HCl, pH 7.5). An equal volume of phenol : chloroform : isoamyl alcohol (25 : 24 : 1, v/v/v) was added, gently mixed and centrifuged at 12,000 rpm (Labofuge 400R, Heraeus Instruments, Heraeus Instruments GmbH, Germany) at 4°C for 10 min. The top supernatant was transferred to a fresh microcentrifuge tube. An equal volume of isopropanol (Merck) was then added, gently mixed and placed in an icebox for 10-30 min to precipitate genomic DNA. The tube was centrifuged at 12,000 rpm for 10 min at 4°C. The DNA pellet was washed with 400 µl of 70% ethanol (Merck), air dried and then resuspended in 50 µl TE buffer. RNA was removed by adding Ribonuclease A (1 mg/ml) (Invitrogen, U.S.A.) to give a

concentration of 10 μg RNase/ml sample and the tube was incubated at 37°C for 30 min.

Genomic DNA was detected in 1% agarose gel electrophoresis, stained with ethidium bromide (1 mg/ml) and examined under UV transilluminator (BioRad). The concentration of DNA was measured by SmartSpec TM 3000 spectrophotometer at 260 nm (BioRad), and the purity of DNA was calculated from the ratio of optical density at 260/280. The conversion factor for determination of DNA concentration is 1 OD_{260} = 50 μg/ml of double stranded DNA. Then, DNA solution was maintained at -20°C until use.

3.11.2 Amplification of 18S rRNA gene

The 18S rRNA gene was amplified using the polymerase chain reaction (PCR). The 18S ribosomal RNA gene amplification was performed using NS1, NS4, SR8R and NS8 oligonucleotides as forward and reverse primers, respectively, to obtain the gene product (1,800 bp) (Figure 3.3 and Table 3.1). The PCR amplification reaction was performed in 50 μl mixtures containing 50 ng of fungal DNA, 5 μl of 10X reaction buffer (10 mM KCL, 20 mM Tris-HCl, pH 8.8, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100), 200 μM of each dATP, dCTP, dGTP and dTTP, 10 μM of each primer, 0.75 unit of *Taq* DNA polymerase and adjusted volume to 50 μl with deionized water. The program of amplification consisted of 1 cycle of 95°C for 3 min; 35 cycles of 95°C for 1 min, 54°C for 1.30 min, 72°C for 2 min; and the final cycle of 72°C for 10 min. The PCR reactions were carried out in the automated thermal cycle (Bioscience Technologies Division, U.S.A.).

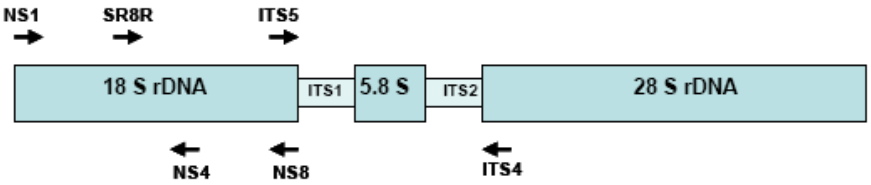

Figure 3.3 Map of oligonucleotide primers for 18S rDNA and ITS region amplification. The arrowheads represent the 3′ end of each primer. Source: Kwang and Kim (1999).

Table 3.1 Nucleotide sequences of PCR primers used for amplification of 18S rRNA gene.

Name	Sequence (5'-3')	Target region[a]	Reference
NS1	GTAGTCATATGCTTGTCTC	SSU 20-38	White *et al*. 1990
SR8R	GAACCAGGACTTTTACCTT	SSU 732-749	Vilgalys *et al*. 1990
NS4	CTTCCGTCAATTCCTTTAAG	SSU 1150-1131	White *et al*. 1990
NS8	TCCGCAGGTTCACCTACGGA	SSU 1788-1768	White *et al*. 1990

[a] *Saccharomyces cerevisiae* numbering

3.11.3 Detection of PCR products by agarose gel electrophoresis

Agarose gel electrophoresis is a standard method used to separate, identify and purify DNA fraction. Agarose gel was prepared at a concentration of 1% (w/v) in 1X TBE buffer (Appendix A6.5), melted in microwave oven until completely dissolved, and then poured into gel box with an appropriate comb. Five microliters of PCR-amplified product were thoroughly mixed with 6X loading buffer (Appendix A6.6). The mixture was loaded into the submarine 1% agarose gel and electrophoresis

was carried out at constant 100 volts until the bromphenol blue dye reached about 2 cm. from the lower edge of the gel, then the electrophoresis was stopped. One thousand base pair DNA ladder (Fermentas, Sovania) was used as standard markers to determine the molecular size of DNA fragments.

After electrophoresis, the agarose gel was stained with ethidium bromide by soaking the gel in a solution containing 10 μg/ml of ethidium bromide and visualized under UV transilluminator (BioRad). The agarose gel was photographed for being reference.

3.11.4 Purification of PCR products

The single band of the DNA-amplified product as estimated size was purified throughout the Gel/PCR DNA Fragments Extraction Kit (Geneaid). The DNA band of the expected size visualized under the UV light was cut from the gel by a clean blade and placed into a new 1.5 ml microcentrifuge tube. The gel matrix that did not contain DNA material was trimmed off to obtain the minimum volume of the gel. Then, the PCR products were purified by using the Gel/PCR DNA Fragments Extraction Kit (Geneaid) according the manufacturer's instruction.

3.11.5 Sequencing of 18S rRNA gene

Sequencing of 18S rRNA gene was performed using NS1, NS4, SR8R and NS8 primers (Table 2), and Terminator Ready Reaction kit version 2.0 (Perkin Elmer, U.S.A.) in combination with an automated sequencing system. The gene was amplified using thermal cycle. An estimated amount of 100 ng of DNA was used for each reaction together with 5 pmol of each primer, 4 μl of ready reaction mix

deionized water to attain a 10 µl final volume. The same primers were used as previous PCR amplification. The thermal profile consisted of 25 cycles of 10 sec at 96°C, 5 sec at 50°C, and 4 min at 60°C. The cycle sequencing was performed in the thermal cycle (BioRad). The PCR mixtures were spun down briefly before DNA precipitation. The sequencing DNA fragments were precipitated by adding 16 µl of deionized water and 64 µl of 95% ethanol. The tube was vortexed briefly, incubated at 4°C for 15 min and then spun at 12,000 rpm for 20 min at 4°C. The DNA pellet was washed with 300 µl of 70% ethanol, centrifuged at 12,000 rpm for 20 min at 4°C, and discarded the supernatant. The DNA pellet was dried at room temperature in the dark. Then, sequencing was performed using ABI377 Automated DNA sequencer (Perkin Elmer, U.S.A.).

3.11.6 Alignment of DNA sequences

Nucleotide sequences of 1,800 bp were aligned using ClustualW at the European Bioinformatics Institute (EBI) (http://www.ebi.ac.uk/Tools/clustalw/) and compared to those available in GenBank databases using Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI: http://www.ncbi.nlm.nih.gov) for finding regions of local similarity between sequences.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Collection of straw mushroom specimens

Fresh fruiting bodies of straw mushroom specimens cultivated in different locations in North-eastern Thailand were collected during May to July 2008, from local markets in North-eastern Thailand. A total of 11 straw mushroom specimens from different locations for cultivation and collection were summarized in Table 4.1 and Figure 4.1.

Table 4.1 Locations of straw mushroom specimens cultivation and collection in North-eastern Thailand.

Mushroom extract code	Location of straw mushroom	
	collection	cultivation
MC131	Phimai, Nakhon Ratchasima	Nakhon Ratchasima
MC132	Nong Bun Mak, Nakhon Ratchasima	Nakhon Ratchasima
MC133	Maung, Surin	Surin
MC134	Nang Rong, Buriram	Buriram
MC138	Chum Phae, Khon Kaen	Khon Kaen
MC139	Maung, Kalasin	Kalasin
MC140	Maung, Nakhon Ratchasima	Buriram
MC144	Thep Sathit, Chaiyaphum	Nakhon Ratchasima
MC145	Kosum Phisai, Maha Sarakham	Khon Kaen
MC167	Maung, Sakon Nakhon	Sisaket
MC168	Warinchumrab, Ubon Ratchathani	Sisaket

Figure 4.1 Fresh fruiting bodies of straw mushroom specimens collected from local markets in North-eastern Thailand. A and B, MC131 from Phimai, Nakhon Ratchasima; C, MC132 from Nong Bun Mak, Nakhon Ratchasima; D, MC133 from Maung, Surin; E, MC134 from Nang Rong, Buriram; F, MC138 from Chum Phae, Khon Kaen.

Figure 4.1 (Continued) Fresh fruiting bodies of straw mushroom specimens collected from local markets in North-eastern Thailand. G, MC139 from Maung, Kalasin; H, MC140 from Nong Ki, Buriram; I, MC144 from Thep Sathit, Chaiyaphum; J, MC145 from Kosum Phisai, Maha Sarakham; K, MC167 from Maung, Sakon Nakhon; L, MC168 from Warinchumrab, Ubon Ratchathani.

4.2 Morphological characteristics of straw mushroom fruiting bodies

A total of 11 straw mushroom specimens which cultivated in 6 provinces: Nakhon Ratchasima, Surin, Buriram, Khon Kaen, Kalasin and Sisaket Provinces in North-eastern Thailand were confirmed for their identification relied on morphological characteristics. These specimens were mainly in the mature stage and elongation stage.

At the mature stage, the whole fruiting body was divided into three regions, namely cap, stalk and volva. The volva was a thin sheet of interwoven hyphae around the bulbous base of the stalk. It was fleshy, white and cup shaped. The stalk was attached to the center of the lower surface of the cap and connected it to the volva. The length of the stalk varies according to the size of the cap. The length was usually about 3-8 cm, and the diameter was about 0.5-1.5 cm. It was white, fleshy and without an annulus. The cap was fully expanded and circular, with an entire margin and a smooth surface which was dark grey in color at the center but light grey near the margin. The diameter was about 6-12 cm. The lower surface of the cap bears many gills. The number varies from 280-380. They were straight and had an entire margin. The gills were not touching the stalk, but were apart from it about 1 mm. The basidiospore morphology of the straw mushrooms was additionally studied using light microscope. Usually four basidiospores attached to each basidium. The shape of mature basidiospores was most asymmetric with a slight tendency to be egg-shaped. The average in length of the egg-shaped spores was 7-9 μm; the width of the widest end was 5-6 μm; the narrow end was 3-4 μm. The spores were with smooth but thick covering, at the narrow end, there was a short, triangular spine sticking out. When viewed with transmitted light microscope, the color of the cell wall and the cell

content varies from transparent, light yellow to pink and dark brown. At the elongation stage, it was similar in shape to the mature one except that the cap was wide ovate. The stalk was stretched out at almost full length at this stage.

For identification relied on morphological characteristics, all 11 straw mushroom specimens had similar morphology (Figure 4.2), belonged to *Volvariella volvacea*.

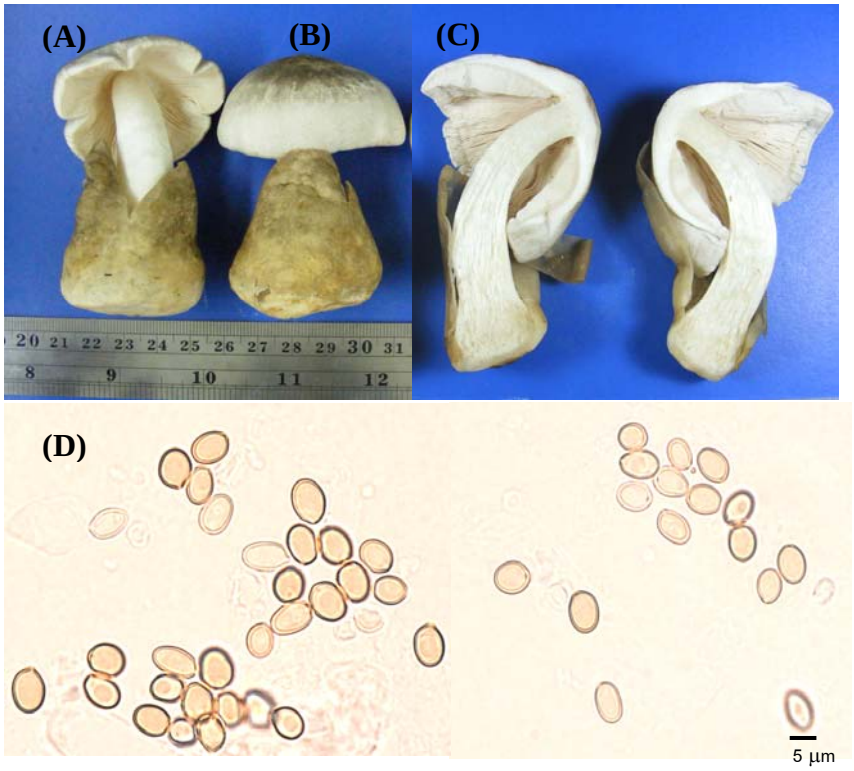

Figure 4.2 Morphology of straw mushroom specimen MC131. A to C, straw mushroom at mature stage; C, cut view of straw mushroom at mature stage; D, basidiospores.

4.3 Determination of lectins accumulated in straw mushroom specimens

Accumulation of lectins in crude extracts from fruiting bodies of 11 straw mushroom specimens which cultivated from different locations in North-eastern Thailand, were detected by hemagglutination assay using rabbit red blood cells. Crude lectins were extracted from 3 different parts (cap, stalk and whole fruiting body) of dried fruiting bodies. Lectins in crude extracts (1:10 dilution) from cap, stalk and whole fruiting body displayed different hemagglutination titers in the range of 256 to 4,096, 256 to 2,048 and 256 to 4,096 respectively (Table 4.2).

Table 4.2 Hemagglutination titers of lectins accumulated in fruiting bodies of straw mushroom when tested against rabbit red blood cells.

Extract code	Location of straw mushroom cultivation	Hemagglutination titer		
		Cap	Stalk	Whole fruiting body
MC131	Nakhon Ratchasima	1024	1024	1024
MC132	Nakhon Ratchasima	1024	1024	1024
MC133	Surin	512	512	4096
MC134	Buriram	2048	2048	2048
MC138	Khon Kaen	1024	1024	1024
MC139	Kalasin	1024	1024	1024
MC140	Buriram	128	256	128
MC144	Nakhon Ratchasima	1024	512	1024
MC145	Khon Kaen	1024	1024	1024
MC167	Sisaket	2048	2048	2048
MC168	Sisaket	256	256	256

Hemagglutination tests of straw mushroom extracts against rabbit red blood cells were shown in Appendix C. In this study, the high variation of lectin accumulation could be observed in each individual straw mushroom from different cultivation areas. This demonstrated clearly that geography was highly effected to lectin occurrence in the particular specimens. Moreover, the fruiting body size and age might give a high variation of the lectin accumulation.

4.4 Determination of biological properties of straw mushroom lectins

4.4.1 Antimicrobial activity test

Crude lectin extracts were then tested for antimicrobial activity using the agar disc diffusion assay. From the agar disc diffusion assay results, only 5 straw mushroom extracts: MC131, MC133, MC134, MC145 and MC168, showed antimicrobial activity (Table 4.3 and Figures 4.3 and 4.4). Concentrations of straw mushroom extracts that contained antibacterial activity, were calculated with standard curve of standard streptomycin (Appendixes B2-B5). Crude extracts of MC131 and MC133 displayed inhibitory activity against *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922 at concentrations of 0.09 and 0.123, 0.057 and 0.053, and 0.116 and 0.128 μg/μl, respectively, compared to standard streptomycin. Extracts of MC134 and MC145 had inhibitory activity with the Gram-positive bacterium, *Staphylococcus aureus* ATCC 29213 at concentrations of 0.066 and 0.065 μg/μl respectively compared to standard streptomycin (Table 4.5). Furthermore, crude lectin extracts of MC145 and MC168 displayed antifungal activity. MC145 and MC168 extracts could inhibit the growth of *Aspergillus niger* ATCC 6275 at 1.65 and 1.46 times narrower than inhibition zone

diameter of standard nystatin (100 units) respectively. MC168 extract also showed considerable activity against *Candida albican* ATCC 10231 at 1.61 times narrower than inhibition zone diameter of standard nystatin (100 units) whereas it showed positive result against *Penicillium funiculosum* ATCC 36839 at 1.692 times wider than inhibition zone diameter of standard nystatin (100 units).

Table 4.3 Antimicrobial activity of straw mushroom extracts using the agar disc diffusion assay.

Mushroom extract code	Inhibition zone diameter (mm)								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
MC131	-	11.5	11	10.3	-	-	-	-	-
MC132	-	-	-	-	-	-	-	-	-
MC133	-	13.5	11.5	10.5	-	-	-	-	-
MC134	-		-	11	-	-	-	-	-
MC138	-	-	-	-	-	-	-	-	-
MC139	-	-	-	-	-	-	-	-	-
MC140	-	-	-	-	-	-	-	-	-
MC144	-	-	-	-	-	-	-	-	-
MC145	-	-	-	11	11.5	-	-	-	-
MC167	-	-	-	-	-	-	-	-	-
MC168	-	-	-	-	13	13	22	-	-

1: *Bacillus cereus* ATCC 11778, 2: *Bacillus subtilis* ATCC 6633, 3: *Escherichia coli* ATCC 25922,
4 : *Staphylococcus aureus* ATCC 29213, 5: *Aspergillus niger* ATCC 6275,
6 : *Candida albican* ATCC 10231, 7 : *Penicillium funiculosum* ATCC 36839,
8 : *Saccharomyces cerevisiae* ATCC 9896, 9: *Saccharomyces cerevisiae* ATCC 18824

Table 4.4 Antimicrobial activity of standard streptomycin (10 μg/μl) and nystatin (100 units) against bacteria and fungi.

Antibiotics	Inhibition zone diameter (mm)								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Streptomycin	16	16	11	13	-	-	-	-	-
Nystatin	-	-	-	-	19	21	16	21	21

1: *Bacillus cereus* ATCC 11778, 2: *Bacillus subtilis* ATCC 6633, 3: *Escherichia coli* ATCC 25922,
4 : *Staphylococcus aureus* ATCC 29213, 5: *Aspergillus niger* ATCC 6275,
6 : *Candida albican* ATCC 10231, 7 : *Penicillium funiculosum* ATCC 36839,
8 : *Saccharomyces cerevisiae* ATCC 9896, 9: *Saccharomyces cerevisiae* ATCC 18824

Table 4.5 Concentration of antibacterial activity in straw mushroom extracts comparing with standard curve of streptomycin tested against bacteria.

Mushroom extract code	Concentration (μg/μl)			
	1	**2**	**3**	**4**
MC131	-	0.090	0.057	0.116
MC133	-	1.235	0.053	0.128
MC134	-	-	-	0.066
MC145	-	-	-	0.065

1: *Bacillus cereus* ATCC 11778, 2: *Bacillus subtilis* ATCC 6633,
3: *Escherichia coli* ATCC 25922, 4 : *Staphylococcus aureus* ATCC 29213

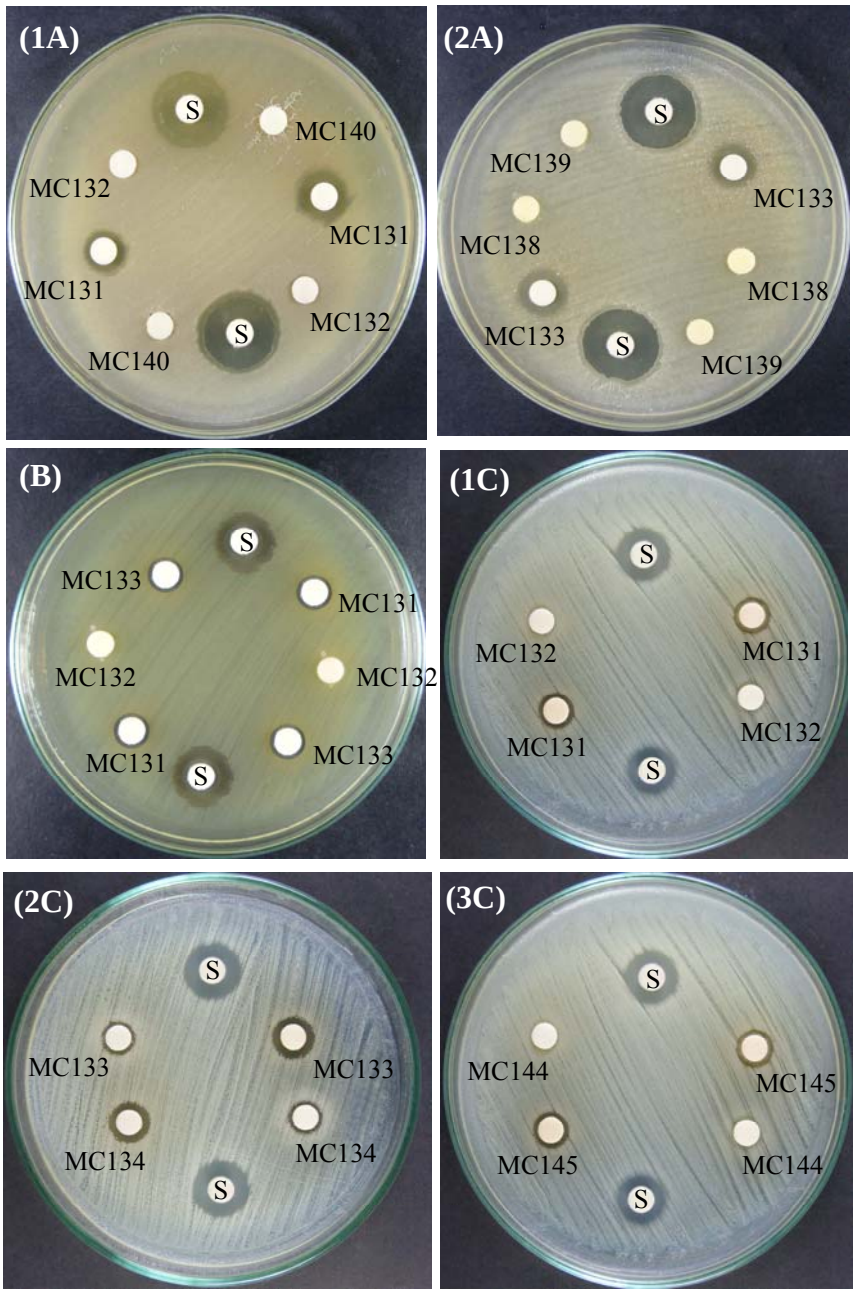

Figure 4.3 Antibacterial activity of crude lectin extracts of MC131, MC133 and MC145 against *Bacillus subtilis* ATCC 6633 (1A and 2A), *Escherichia coli* ATCC 25922 (B) and *Staphylococcus aureus* ATCC 29213 (1C, 2C and 3C) after 24 h incubation. The inhibition of streptomycin (10 μg/μl) (S) showed as the positive control.

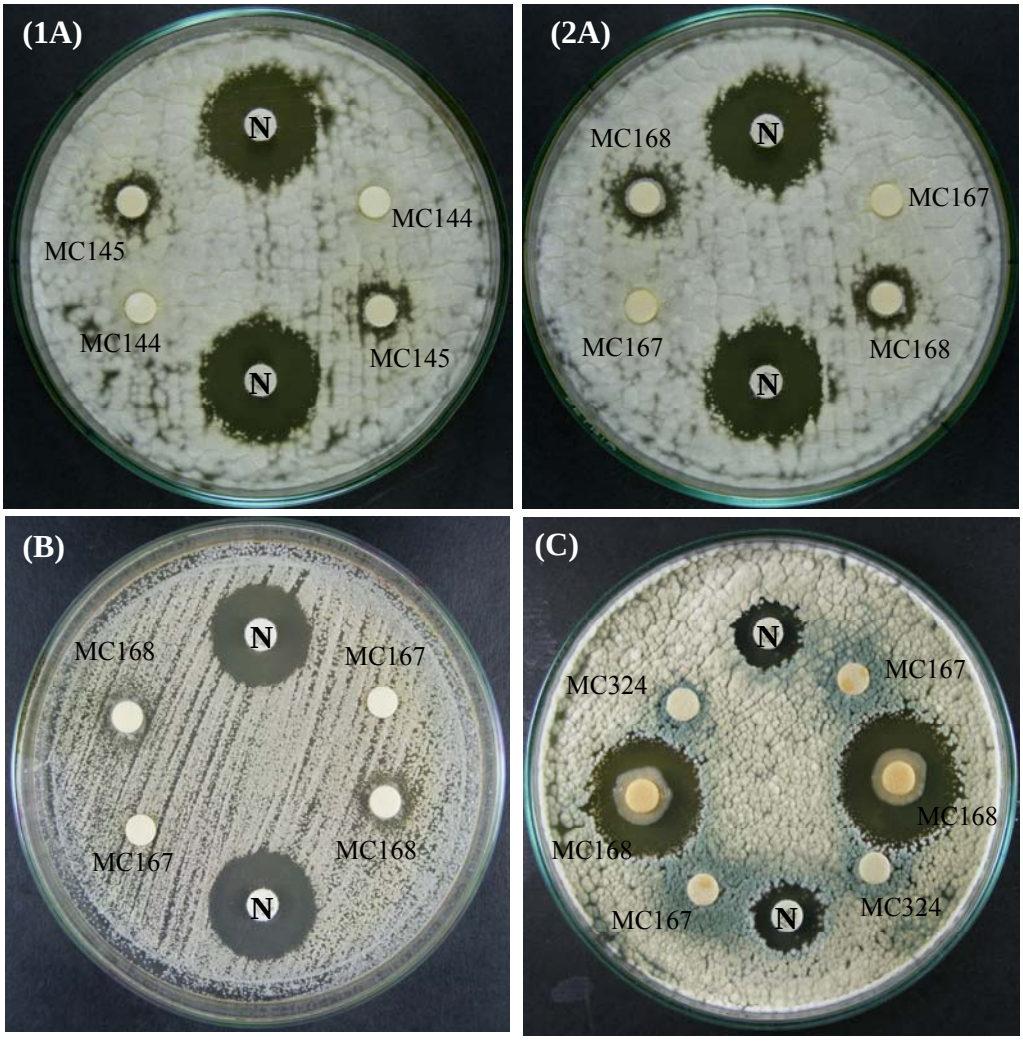

Figure 4.4 Antifungal activity of crude lectin extracts MC145 and MC168 against *Aspergillus niger* ATCC 6275 (1A and 2A), *Candida albican* ATCC 10231 (B) and *Penicillium funiculosum* ATCC 36839 (C) after 48 h incubation. The inhibition of nystatin (100 units) (N) showed as the positive control.

4.4.2 Cytotoxicity test against human cancer cells

Crude lectin extracts of 11 straw mushroom specimens were submitted to cytotoxicity tests against human epidermoid carcinoma (KB) and human cervical carcinoma (HeLa). African green monkey kidney epithelial (Vero) cells were used as the representative of normal cells. Results from cytotoxic activity test showed that 8 straw mushroom extracts: MC131, MC133, MC134, MC138, MC139, MC144, MC145 and MC167 exhibited cytotoxicity against KB cell with IC_{50} values ranging from 1.40 to 13.68 μg/ml whereas the cytotoxicity toward vero cell was observed on crude extracts of MC131, MC132, MC133, MC134, MC138, MC139, MC144, MC145 and MC167 with IC_{50} values ranging from 5.63 to 20.15 μg/ml. Only MC134 extract showed cytotoxicity against HeLa cell with IC_{50} value of 21.38 μg/ml (Table 4.6).

Although the straw mushroom extracts exhibited cytotoxicity against vero cell but this result could not stress directly that these lectins are toxic to all normal cell lines. Thus, testing with other normal cell line e.g. fibroblast is need. This study indicated that the popular edible mushroom, straw mushroom which was available in high quantity in the local markets of North-eastern Thailand, showed cytotoxicity toward both human epidermoid carcinoma (KB) and human cervical carcinoma (HeLa) cells. This is good for drug development since epidermoid carcinoma and cervical carcinoma currently appear to be a major cause of cancer in patients in Thailand.

Table 4.6 Cytotoxicity test of straw mushroom extracts against human epidermoid carcinoma (KB), human cervical carcinoma (HeLa) and African green monkey kidney epithelial (Vero) cells by MTT colorimetric assay.

Extract code	Location of straw mushroom cultivation	IC_{50} (μg/ml)		
		KB	Hela	Vero
MC131	Nakhon Ratchasima	4.80±0.30	40.89±4.31	7.00±0.37
MC132	Nakhon Ratchasima	51.34±0.32	47.06±3.90	20.15±0.36
MC133	Surin	1.40±0.15	61.68±4.70	15.44±1.36
MC134	Buriram	4.06±0.34	21.39±2.62	5.63±0.26
MC138	Khon Kaen	5.08±0.39	>100	10.78±2.19
MC139	Kalasin	11.96±0.96	>100	14.76±1.49
MC140	Buriram	44.34±2.95	82.74±3.20	40.11±0.91
MC144	Nakhon Ratchasima	13.68±3.71	82.74±3.18	17.05±0.95
MC145	Khon Kaen	10.40±2.43	45.99±5.64	12.26±2.26
MC167	Sisaket	7.30±0.87	66.20±2.40	6.32±0.30
MC168	Sisaket	>100	>100	>100

KB: Human epidermoid carcinoma cell line

HeLa: Human cervical carcinoma cell line

Vero: African green monkey kidney epithelial cell line

IC_{50} value less than 30 μg/ml was designated as cytotoxicity

4.5 Selection of straw mushroom lectin according to their biological properties

According to results of biological activity tests, the straw mushroom specimen MC131 was selected for further purification and characterization of its lectin according to its capability to perform the incidence of lectin accumulation in its fruiting bodies, its ability antimicrobial and cytotoxic activities.

4.6 Purification of the selected straw mushroom lectin

4.6.1 Preliminary study of purification methods for the selected straw mushroom lectin

For purification of straw mushroom lectin, different types of affinity chromatography using porcine stomach mucin (PSM)-Sepharose 4B column, ion exchange chromatography using DEAE-Sepharose column and CM-Sepharose column, were investigated. It was found that the ion exchange chromatography on DEAE-Sepharose column was found to be suitable for purification of straw mushroom specimen MC131.

4.6.2 Purification of the selected straw mushroom lectin by ion-exchange chromatography

Dried fruiting bodies (100 g) of straw mushroom specimen MC131 cultivated from Phimai District, Nakhon Ratchasima Province were homogenized in 10 times (w/v) of 10 mM PBS, pH 7.4 containing 1 mM benzamidine, 0.1% 2-mercaptoethanol and 1.5% (w/v) polyvinylpyrrolidone (PVPP). 1.5% (w/v) of

polyvinylpyrrolidone powder was helpfully overcome the problem of polyphenols which had been previously observed to interfere with protein absorbance at 280 nm. Because mushrooms also have high levels of phenolic compounds as plants have. The crude lectin extract was prepared from ammonium sulfate saturation (90% saturation), and then applied on DEAE-Sepharose column (1.5×11 cm). A DEAE-Sepharose column was equilibrated and eluted with 10 mM tris-HCl buffer, pH 7.3, and then stepwise with 0-0.5 M NaCl in the same buffer. Three peaks D1, D2 and D3 were observed by 160 ml 0-0.5 M NaCl step wise elution (Figure 4.5). The D2 peak eluted with 0.2 M NaCl was the peak with hemagglutinating activity (Table 4.7).

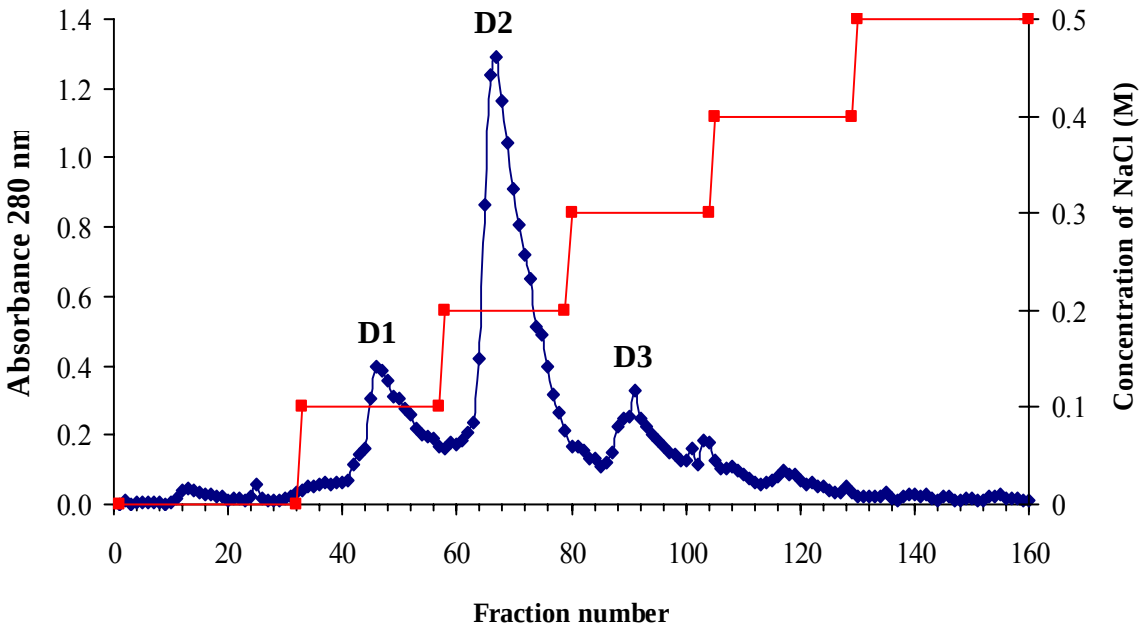

Figure 4.5 Anion exchange chromatography of crude lectin extract from MC131 on DEAE-Sepharose column (1.5×11 cm), which was equilibrated and eluted with 10 mM Tris-HCl buffer, pH 7.3, and then stepwise using the same buffer containing NaCl at concentration 0-0.5 M. The flow rate was 0.4 ml/min.

4.6.3 Purification of the partial purified lectin by gel filtration chromatography

From the first purification of straw mushroom MC131, the fractions containing peak D2 were pooled, concentrated, and applied on Superdex 75 10/300 GL column by Fast protein liquid chromatography (FPLC) on an ÄKTA purifier system. A Superdex 75 column was equilibrated and eluted with 10 mM Tris-HCl, pH 7.3 containing 100 mM NaCl. The hemagglutinating activity was enriched in the main peak G2 (Figure 4.6 and Table 4.7). This peak was collected and represented purified lectin.

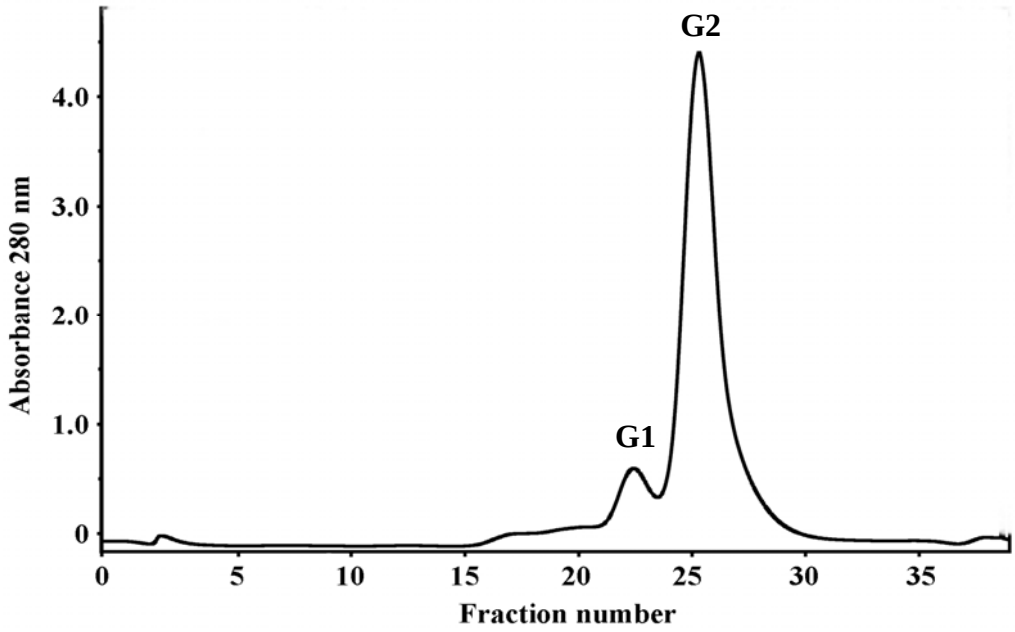

Figure 4.6 Gel filtration of fraction D2 from DEAE-Sepharose column on Superdex 75 column 10/300 GL by FPLC on an AKTA Purifier, which was eluted with 10 mM Tris-HCl, pH 7.3 containing 100 mM NaCl. The flow rate was 0.5 ml/min. Fraction G2 represented the purified lectin.

The maximum purity of 49.27 purification folds of straw mushroom MC131 lectin could be obtained from 100 g of dried fruiting bodies (Table 4.7). The specific hemagglutinating activity of the crude lectin extract was obtained at 2.5 units/mg, but after purification by gelfiltration chromatography on a Superdex 75 column, the specific hemagglutinating activity of lectin was increased up to 123.21 units/mg. From 100 g of dried straw mushroom powder, 9.34 mg of lectin was obtained.

Table 4.7 Summary of yields of straw mushroom MC131 lectin purified from dried fruiting bodies (100 g).

Step	**Yield (mg)**	**Specific hemaggluti-nating activity (units/mg)**	**Total hemaggluti-nating activity (units)**	**Recovery of hemaggluti-nating activity (%)**	**Fold of purifica-tion**
Extraction	6,393	2.50	16,000	100.00	1.00
90% $(NH_4)_2SO_4$ Precipitation	2974.2	5.16	15360	96.00	2.06
DEAE-Sepharose column	2190.24	2.10	4608	28.80	0.84
Superdex 75 column	9.34	123.31	1152	7.20	49.27

4.7 Characterization of the purified straw mushroom lectin

4.7.1 Chemical properties of the purified straw mushroom lectin

4.7.1.1 Molecular weight

Polyacrylamide gel electrophoresis in the presence of SDS was investigated the molecular weight for all purified lectin samples from each step of the purification process. The purified lectin samples, approximately 20 µl was loaded on to the SDS-PAGE (12.5% Gel). Data was shown in Figure 4.7.

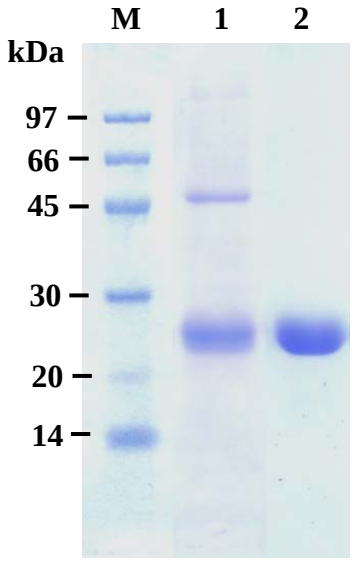

Figure 4.7 SDS-PAGE (12.5% Gel) of purified MC131 lectin. Lanes: M, LMW protein markers (GE Healthcare) comprising phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), bovine carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa) and bovine α-lactalbumin (14 kDa); 1, fraction D2 which was partial purified MC131 lectin from DEAE-Sepharose column; 2, fraction G2 which was purified MC131 lectin from gel filtration column.

MC131 lectin was purified by anion exchange chromatography on DEAE-Sepharose column and gel filtration chromatography on a Superdex 75 column, appeared as a single band with a molecular weight of 25 kDa in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing condition (Figure 4.7). The estimation of molecular weight of native lectin by gel filtration based on the standard markers calculated the molecular weight of lectin as 25 kDa (Appendix 6B). These result indicated that native of MC131 lectin was most likely a monomeric lectin.

4.7.1.2 Isoelectric point (pI)

The isoelectric point (pI) of the purified MC131 lectin was determined by 2D-electrophoresis. The purified MC131 lectin showed a spot in the pH zone near 5.0 (Figure 4.8).

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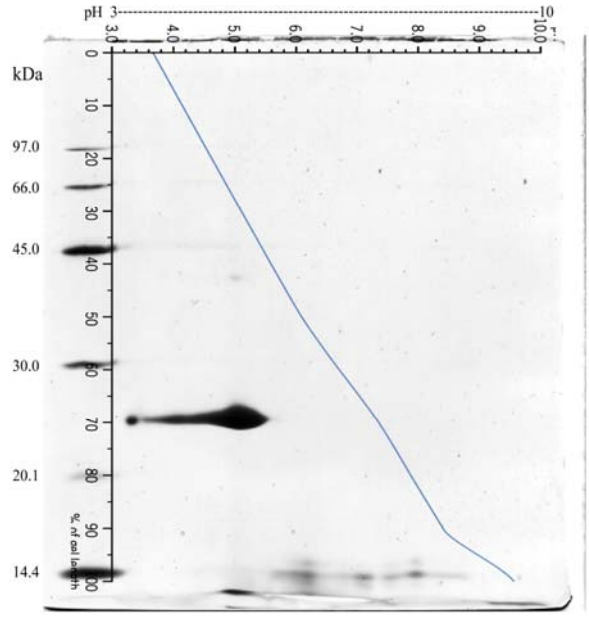


Figure 4.8 2D-PAGE of purified MC131 lectin on 7 cm IPG strip, pH range 3.0-10.0

4.7.1.3 Glycoprotein property

The periodic acid schiff's (PAS) staining was determined glycoprotein property of the lectins. This method indicated that the strong pink color band was a glycosylated protein (Figure 4.9). Thus, MC131 lectin was a glycoprotein comparing with positive control of ribonuclease B (RNase B) which is the glycoprotein from bovine pancreas.

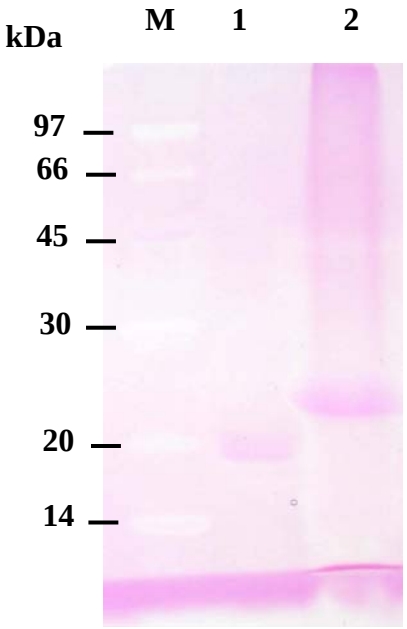


Figure 4.9 Periodic acid schiff's (PAS) assay of MC131 lectin using Ribonuclease B as a positive control. Lane: M, L protein markers (GE Healthcare) comprising phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), bovine carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa) and bovine α-lactalbumin (14 kDa); LMW-SDS markers (GE Healthcare); 1, ribonuclease B from bovine pancreas (Sigma-aldrich); 2, purified MC131 lectin.

4.7.1.4 Amino acid sequence

The purified lectin band (25 kDa) from SDS-PAGE was excised and in-gel digested. The tryptic peptides were analyzed by liquid chromatography-electrospray ionization mass spectrometry (LC/ESI/MS) and subjected to MS/MS ion search in MASCOT search engine. The tryptic peptide sequences of MC131 lectin were shown in Table 4.8.

Table 4.8 Identification of tryptic peptides of lectin from straw mushroom MC131 lectin by LC/ESI/MS.

Tryptic peptide	Observed mass	Calculated mass	Amino acid sequences
T1	2866.3282	2866.4600	ASDDNVFQPVDQLPEDLIPSSIQVLK
T2	-	-	FSGK
T3	1035.5654	1035.5600	YLKLEQDK
T4	1742.8252	1742.8304	LEQDKAYFDWPGFK
T5	1129.4934	1129.5233	AYFDWPGFK
T6	1687.7034	1687.7577	TAIDNYTGEDLSFDK
T7	2139.8482	2139.9743	YDQSTINQQSQEVGAMVDK
T8	-	-	IAK
T9	1531.8034	1531.8035	FLHDAFAAVVDLSK
T10	3213.8662	3213.6193	LAAIILNTFTNLEEESSSGFLQFNTNNVK
T11	1068.4374	1068.4988	KNSSWEYR
T12	1222.5814	1222.5983	TGWWGLTSSTK
T13	1686.0074	1685.9716	KNFAVQIDALELVVK
T14	1557.8774	1557.8766	NFAVQIDALELVVK
T15	-	-	KGFKAPN

The partial peptide sequences of MC131 lectin were then submitted to the protein database (the NCBI Blast database). The sequence similarity with other lectins was observed in Figure 4.10. The results from the NCBI Blast database indicated that MC131 lectin had the highest percentage similarity at 99% with the volvatoxin A2 precursor of *Volvariella volvacea* (AAQ92757) from GenBank database U.S.A. (Figure 4.11)

Sequence alignments of MC131 lectin

T1	ASDDNVFQPVDQLPEDLIPSSIQVLK
T3-T4-T6	YLKLEQDK LEQDKAYFDWPGFK TAIDNYTGEDLSFDK
T5	AYFDWPGFK
T7	YDQSTINQQSQEVGAMVDK
T9-T10-T11	FLHDAFAAVVDLSKLAAIILNTFTNLEEESSSGFL QFNTNNVK KNSSWEYR
T12-T13_T15	TGWWGLTSSTK KNFAVQIDALELVVK KGFKAPN

T1 ASDDNVFQPVDQLPEDLIPSSIQVLK

>**gb|AAQ92757.1|** volvatoxin A2 precursor [Volvariella volvacea]
Length=217

Score = 85.5 bits (194), Expect = 7e-16
Identities = 26/26 (100%), Positives = 26/26 (100%), Gaps = 0/26 (0%)

```
Query  1   ASDDNVFQPVDQLPEDLIPSSIQVLK  26
           ASDDNVFQPVDQLPEDLIPSSIQVLK
Sbjct  19  ASDDNVFQPVDQLPEDLIPSSIQVLK  44
```

T3-T4-T6 YLKLEQDK LEQDKAYFDWPGFK TAIDNYTGEDLSFDK

>**gb|AAQ92757.1|** volvatoxin A2 precursor [Volvariella volvacea]
Length=217

Score = 66.6 bits (161), Expect = 8e-10, Method: Compositional matrix adjust.
Identities = 30/30 (100%), Positives = 30/30 (100%), Gaps = 0/30 (0%)

```
Query  8   KLEQDKAYFDWPGFKTAIDNYTGEDLSFDK  37
           KLEQDKAYFDWPGFKTAIDNYTGEDLSFDK
Sbjct  51  KLEQDKAYFDWPGFKTAIDNYTGEDLSFDK  80
```

Figure 4.10 Sequence similarity of tryptic peptides of MC131 lectin with other lectins from the NCBI database.

T5 AYFDWPGFK

```
>gb|AAQ92757.1|  volvatoxin A2 precursor [Volvariella volvacea]
Length=217

 Score = 35.4 bits (76),  Expect = 0.72
 Identities = 9/9 (100%), Positives = 9/9 (100%), Gaps = 0/9 (0%)

Query  1   AYFDWPGFK  9
           AYFDWPGFK
Sbjct  57  AYFDWPGFK  65
```

T7 YDQSTINQQSQEVGAMVDK

```
>gb|AAQ92757.1|  volvatoxin A2 precursor [Volvariella volvacea]
Length=217

 Score = 35.4 bits (76),  Expect = 0.72
 Identities = 9/9 (100%), Positives = 9/9 (100%), Gaps = 0/9 (0%)

Query  1   AYFDWPGFK  9
           AYFDWPGFK
Sbjct  57  AYFDWPGFK  65

Sbjct  81  YDQSTINQQSQEVGAMVDK  99
```

T9-T10-T11 FLHDAFAAVVDLSKLAAIILNTFTNLEEESSSGFL

QFNTNNVK KNSSWEYR

```
>gb|AAQ92757.1|  volvatoxin A2 precursor [Volvariella volvacea]
Length=217

 Score =  104 bits (260),  Expect = 2e-21, Method: Compositional matrix adjust.
 Identities = 51/51 (100%), Positives = 51/51 (100%), Gaps = 0/51 (0%)

Query  1    FLHDAFAAVVDLSKLAAIILNTFTNLEEESSSGFLQFNTNNVKKNSSWEYR  51
            FLHDAFAAVVDLSKLAAIILNTFTNLEEESSSGFLQFNTNNVKKNSSWEYR
Sbjct  103  FLHDAFAAVVDLSKLAAIILNTFTNLEEESSSGFLQFNTNNVKKNSSWEYR  153
```

Figure 4.10 (Continued) Sequence similarity of tryptic peptides of MC131 lectin with other lectins from the NCBI database.

T12-T13_T15 TGWWGLTSSTK KNFAVQIDALELVVK KGFKAPN

```
>pdb|1VCY|A Chain A, Vva2 Isoform
Length=213

 Score = 70.9 bits (172),  Expect = 4e-11, Method: Compositional matrix adjust.
 Identities = 33/33 (100%), Positives = 33/33 (100%), Gaps = 0/33 (0%)

Query  1    TGWWGLTSSTKKNFAVQIDALELVVKKGFKAPN  33
            TGWWGLTSSTKKNFAVQIDALELVVKKGFKAPN
Sbjct  181  TGWWGLTSSTKKNFAVQIDALELVVKKGFKAPN  213
```

Figure 4.10 (Continued) Sequence similarity of tryptic peptides of MC131 lectin with other lectins from the NCBI database.

```
Volvatoxin A2   MSESELKVNQ  AVLSLVAAAS  DDNVFQPVDQ  LPEDLIPSSI  40
MC131           ---------- -  -- ----- AS  DDNVFQPVDQ  LPEDLIPSSI  22

Volvatoxin A2   QVLK FSGK YL  KLEQDKAYFD  WPGFKTAIDN  YTGEDLSFDK  80
MC131           QVLK---- YL  KLEQDKAYFD  WPGFKTAIDN  YTGEDLSFDK  58

Volvatoxin A2   YDQSTINQQS  QEVGAMVDKI  AKFLHDAFAA  VVDLSKLAAI  120
MC131           YDQSTINQQS  QEVGAMVDK-  --FLHDAFAA  VVDLSKLAAI  95

Volvatoxin A2   ILNTFTNLEE  ESSSGFLQFN  TNNVKKNSSW  EYRVLFSVPF  160
MC131           ILNTFTNLEE  ESSSGFLQFN  TNNVKKNSSW  EYRVLFSVPF  135

Volvatoxin A2   GDNAPSYFYS  LVTTILITAD  EEKTGWWGL   TSSTKKNFAV  200
MC131           GDNAPSYFYS  LVTTILITAD  IEEKTGWWGL  TSSTKKNFAV  175

Volvatoxin A2   QIDALELVVK  KGFKAPN                            217
MC131 lectin    QIDALELVVK  - ------                           185
```

Figure 4.11 Amino acid sequences comparison of MC131 lectin and volvatoxin A2 precursor of *Volvariella volvacea* (AAQ92757) from GenBank database U.S.A. The residues conserved are displayed on black blackground.

4.7.1.5 Sugar specificity

Specificity for carbohydrate binding of MC131 lectin was examined by a hemagglutination inhibition assay. The hemagglutinating activity of purified MC131 lectin with 16 (2^4) hemagglutination units was not specific to 32 sugars tested; *N*-acetyl-D-galactosamine (GalNAc), 2-acetamido-2-deoxy-D-glucopyranose, D-adonitol, amygdalin, L-arabinose, cellobiose, esculin, fructose, fucose, D-galactose, D-glucose, inositol, inulin, lactose, lactitol, maltose, mannitol, D-mannose, D-melezitose, melibiose, methyl-α-D-galactopyranoside, methyl-β-D-galactopyranoside, 4-nitrophynyl-α-D-galactopyranoside, 4-nitrophynyl-β-D-galactopyranoside, raffinose, rhamnose, ribose, salicin, sorbitol, trehalose, D-turanose and D-xylose by the following sugar concentration up to 500 mM. It is likely that the lectin has an affinity for complex carbohydrate structures on the cell surface.

4.7.2 Physical properties of the purified straw mushroom lectin

4.7.2.1 Temperature stability

For temperature stability test, the hemagglutinating activity of MC131 lectin was stable at temperature up to 60°C for 30 min test (Figure 4.12), whereas crude lectin extract of MC131 was stable at 60°C for 1 h test. The hemagglutinating activity of MC131 lectin was less temperature stability than several mushroom lectins such as *Pleurotus citrinopileatus* lectin (Li *et al*., 2008), *Agrocybe cylindracea* lectin (Liu *et al*., 2007), *Armillaria luteo-viren* lectin (Feng *et al*., 2006) and *Ganoderma carpense* lectin (Ngai and Ng, 2004). Its temperature stability was similar to *Ganoderma lucidum* (Thakur *et al*., 2007), *Flammulina velutipes* (Liu *et al*.,

2006) and *Agrocybe aegerita* lectin (Sun *et al*., 2003), which was stable at temperatures up to 60°C.

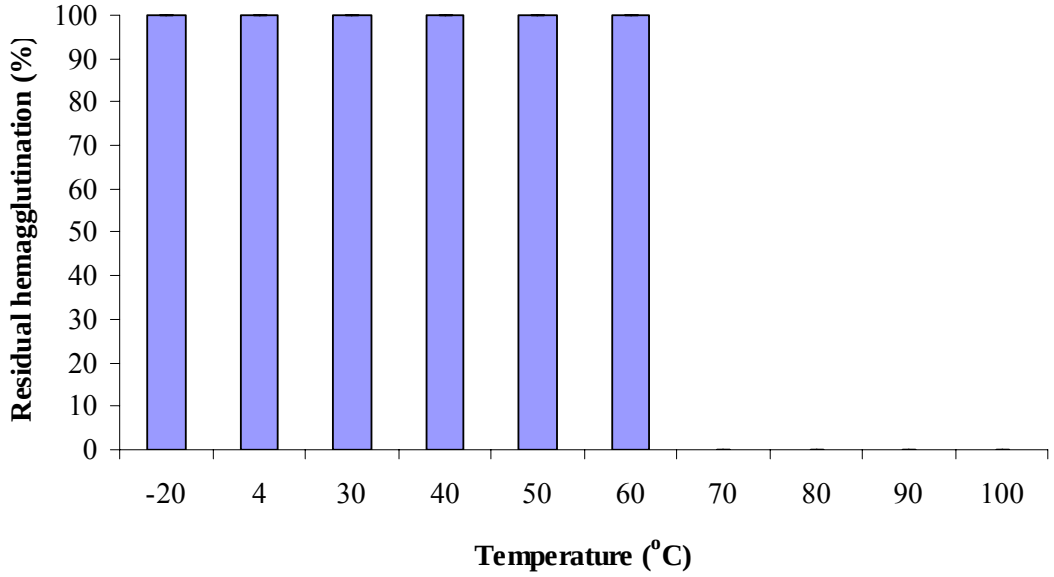


Figure 4.12 Temperature stability of MC131 lectin. Full hemagglutinating activity (100%) corresponded to a titer of 32.

4.7.2.2 pH stability

For pH stability test, the hemagglutinating activity was stable at pH 7 for 18 h test (Figure 4.13). Lectin activity was almost changed after exposure pH between 2-6 or pH between 8-12. Thus it appeared that the activity of MC131 lectin was remained at neutral pH, and rapidly disappeared after exposure to low pH and high pH treatments, while the hemagglutinating activity of crude lectin extract MC131 was stable at pH 5-12 for 18 h test.

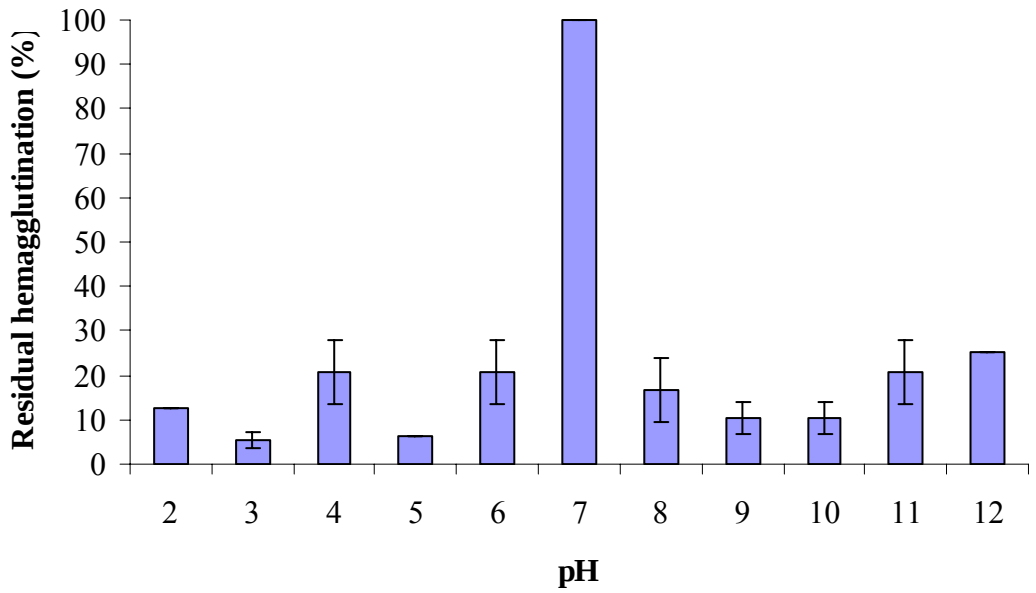


Figure 4.13 The pH stability of MC131 lectin. Full hemagglutinating activity (100%) corresponded to a titer of 64.

4.7.3 Biological properties of the purified straw mushroom lectin

4.7.3.1 Antimicrobial activity

The antimicrobial activity of MC131 lectin was tested against *Bacillus cereus* ATCC 11778*, Bacillus subtilis* ATCC 6633*, Escherichia coli* ATCC 25922*, Staphylococcus aureus* ATCC 29213*, Aspergillus niger* ATCC 6275*, Candida albican* ATCC 10231*, Penicillium funiculosum* ATCC 36839*, Saccharomyces cerevisiae* ATCC 9896 and *Saccharomyces cerevisiae* ATCC 18824 using the agar disc diffusion assay. The result showed that MC131 lectin did not exhibit antimicrobial activity.

4.7.3.2 Cytotoxicity test against cancer cells

. For cytotoxic activity test, MC131 lectin showed the highest cytotoxicity against human epidermoid carcinoma (KB), human cervical carcinoma (HeLa) and African green monkey kidney epithelial (Vero) cells with the IC_{50} value of 0.58, 0.70 and 0.23 μg/ml, respectively (Table 4.9). The IC_{50} value less than 4 μg/ml was designated as cytotoxicity.

Table 4.9 Cytotoxic activity of MC131 lectin against human epidermoid carcinoma (KB), human cervical carcinoma (HeLa) and African green monkey kidney epithelial (Vero) cells by MTT colorimetric assay.

Mushroom lectin	IC_{50} (μg/ml)		
	KB	Hela	Vero
Crude extract MC131	4.80±0.30	40.89±4.31	7.00±0.37
Purified MC131 lectin	0.58±0.087	0.70±0.023	0.23±0.022

KB: Human epidermoid carcinoma cell line

HeLa: Human cervical carcinoma cell line

Vero:African green monkey kidney epithelial cell line

IC_{50} value less than 4 μg/ml was designated as cytotoxicity

4.8 Preliminary study of the selected straw mushroom lectin structure

4.8.1 Crystallization of the selected straw mushroom lectin

4.8.1.1 Determination of crystallization conditions using screening kit

The purified MC131 lectin was dissolved in 10 mM Tris-HCl, pH 8.0 at a concentration of 25 mg/ml for crystallization. Initial crystallization screening was performed using the microbatch under oil technique with Hampton Research Crystal Screen HR2-110 and HR2-112 at 18°C. Results from manually prepared microbatch crystallization showed the promising 4 conditions (condition 10, 28, 40 and 42 from Crystal Screen HR2-110) and 1 condition (condition 22 from Crystal Screen HR2-112) which provided needle-like or plate-like crystals within 1 day of incubation whereas the condition 13 from Crystal Screen HR2-112 produced the small plate-like crystals within 3 days of incubation. Thereafter blank conditions were set-up comparatively to confirm that the morphology observed belonging to lectin crystallization. The promising 6 conditions provided needle-like and plate-like crystals as described in Table 4.10.

Table 4.10 Observation in a microbatch under oil experiment using Crystal Screen HR2-110 and HR2-112 reagent kit (Hampton Research, California, U.S.A.).

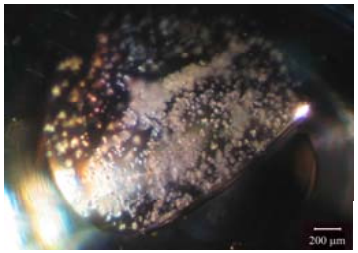
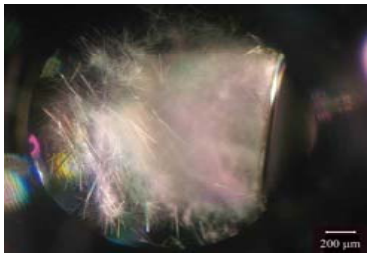
Crystallization-screening reagent kits	Condition	Precipitant composition	Days of incubation	Crystal appearance
Crystal Screen HR2-110	10	30% (w/v) Polyethylene glycol 4,000, 0.2 M ammonium acetate in 0.1 M sodium acetate trihydrate, pH 4.6	1	 Small needle-like crystals
Crystal Screen HR2-110	28	30% (w/v) polyethylene glycol 8,000, 0.2 M sodium acetate trihydrate in 0.1 M sodium cacodylate trihydrate, pH 6.5	1	 Large needle-like crystals

Table 4.10 (Continued) Observation in a microbatch under oil experiment using Crystal Screen HR2-110 and HR2-112 reagent kit (Hampton Research, California, U.S.A.).

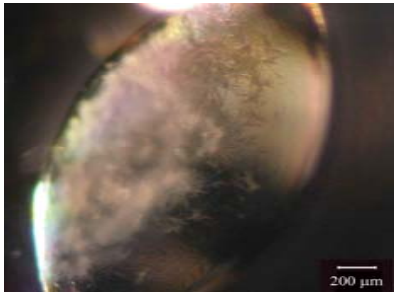
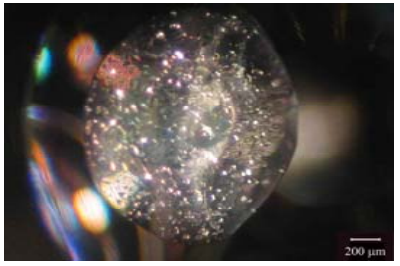
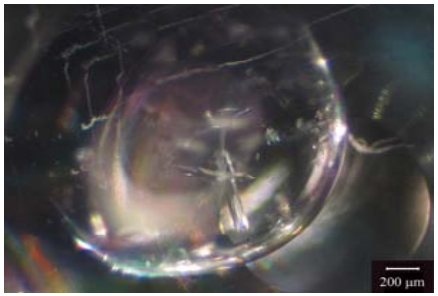
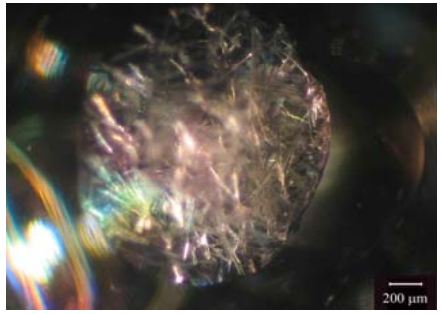
Crystallization-screening reagent kits	Condition	Precipitant composition	Days of incubation	Crystal appearance
Crystal Screen HR2-110	40	20% (v/v) 2-propanol, 20%(w/v) polyethylene glycol 4,000 in 0.1 M sodium citrate tribasic dehydrate, pH 5.6	1	 Small needle-like crystals
Crystal Screen HR2-110	42	20% (w/v) polyethylene glycol 8,000 and 0.05 M potassium phosphate monobasic	1	 Small plate-like crystals

Table 4.10 (Continued) Observation in a microbatch under oil experiment using Crystal Screen HR2-110 and HR2-112 reagent kit (Hampton Research, California, U.S.A.).

Crystallization-screening reagent kits	Condition	Precipitant composition	Days of incubation	Crystal appearance
Crystal Screen HR2-112	13	30% (w/v) polyethylene glycol monomethyl ether 2,000, 0.2 M ammonium sulfate in 0.1 M sodium acetate trihydrate, pH 4.6	3	 Large plate-like crystals
Crystal Screen HR2-112	22	12% (w/v) polyethylene glycol 20,000 in 0.1 M MES monohydrate, pH 6.5	1	 Large needle-like crystals

All 6 conditions from the initial microbatch were further tested by hanging drop vapor diffusion method using the commercial reagent. After 1 week of incubation, 3 out of 6 conditions gave needle-like crystals (condition 40 from Crystal Screen HR2-110 and 22 from Crystal Screen HR2-112) and plate-like crystals (condition 13 from Crystal Screen HR2-112) from the equilibrated 25 mg/ml of the purified lectin at 18°C. Therefore the 3 conditions (condition 40 from Crystal Screen HR2-110 and condition 13 and 22 from Crystal Screen HR2-112) were selected for further optimization of the lectin crystal growth.

4.8.1.2 Optimization of crystal growth conditions

Based on the results of crystallization screening, the 3 conditions (condition 40 from Crystal Screen HR2-110 and condition 13 and 22 from Crystal Screen HR2-112) were selected for further optimization of the lectin crystal growth using hanging drop vapor diffusion method. Through optimization, the purified lectin concentration of 5 mg/ml was used.

For optimization of the condition 40 from Crystal Screen HR2-110; the buffer condition; 20% (v/v) 2-propanol, 20% (w/v) polyethylene glycol 4,000 (PEG 4,000) in 0.1 M sodium citrate tribasic dehydrate, pH 5.6 were adjusted. The concentrations of 2-propanol and PEG 4,000 were studied. A grid of 24 conditions of 5%, 15%, 20% and 25% 2-propanol, and PEG 4,000 concentrations of 6, 12, 16, 18 20 and 26% in 0.1 M sodium citrate tribasic dehydrate, pH 5.6 were prepared (Table 4.11).

Table 4.11 Sparse matrix for optimization of the condition 40 for MC131 lectin crystal growth using hanging drop vapor diffusion method.

Concentration of 2-propanol	Concentration of PEG 4,000 (w/v)					
	6%	12%	16%	18%	20%	26%
5	5%	5%	5%	5%	**5%**	5%
	6%	12%	16%	18%	**20%**	26%
15	15%	15%	15%	15%	15%	15%
	6%	12%	16%	18%	20%	26%
20	20%	20%	20%	20%	20%	20%
	6%	12%	16%	18%	20%	26%
25	25%	25%	25%	25%	25%	25%
	6%	12%	16%	18%	20%	26%

Results from optimization of the condition 40 for the lectin crystals growth showed that the plate-like crystals obtained from the equilibrated 5 mg/ml of purified lectin under the condition of 5% (v/v) 2-propanol, 20% (w/v) PEG 4,000 in 0.1 M sodium citrate tribasic dehydrate, pH 5.6 at 18°C after 1 month of incubation (Figure 4.14A) and after 2 months of incubation (Figure 4.14B). The plate-like crystals from this condition was not suitable for X-ray diffraction analysis.

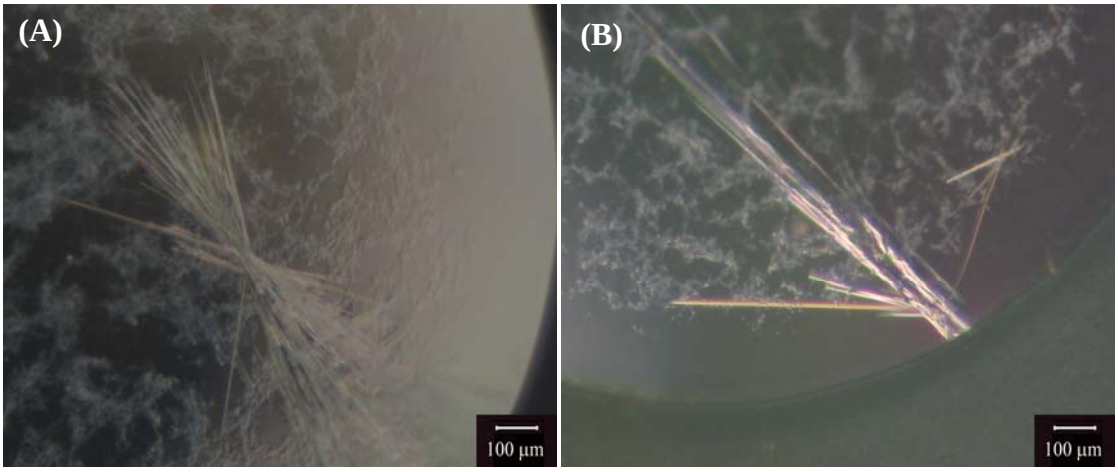


Figure 4.14 Crystals of MC131 lectin grown in hanging drop method using the condition: 5% (v/v) 2-propanol, 20% (w/v) polyethylene glycol 4,000 in 0.1 M sodium citrate tribasic dehydrate, pH 5.6 at 18°C after 1 month of incubation (A) and after 2 months of incubation (B).

For optimization of the condition 13 from Crystal Screen HR2-112; the buffer condition; 30% (w/v) polyethylene glycol monomethyl ether 2,000 (PME 2,000), 0.2 M ammonium sulfate in 0.1 M sodium acetate trihydrate, pH 4.6 were investigated. The concentrations of PME 2,000 and the different buffers were studied. A grid of 24 conditions of 14%, 18%, 22%, 26%, 30% and 34% PME 2,000, and the buffer; 0.1 M acetate, pH 4.6, 0.1 M citrate, pH 5.0, 0.1 M citrate, pH 5.5, and 0.1 M MES, pH 6.0 were prepared (Table 4.12).

Table 4.12 Sparse matrix for optimization of the condition 13 for MC131 lectin crystal growth using hanging drop vapor diffusion method.

Buffer	Concentration of PME 2,000 (w/v)					
	14%	18%	22%	26%	30%	34%
0.1 M Acetate, pH 4.6	4.6	**4.6**	4.6	**4.6**	4.6	4.6
	14%	**18%**	22%	**26%**	30%	34%
0.1 M Citrate, pH 5.0	5.0	5.0	5.0	5.0	5.0	5.0
	14%	18%	22%	26%	30%	34%
0.1 M Citrate, pH 5.5	5.5	5.5	5.5	5.5	5.5	5.5
	14%	18%	22%	26%	30%	34%
0.1 M MES, pH 6.0	6.0	6.0	6.0	6.0	6.0	6.0
	14%	18%	22%	26%	30%	34%

Results from optimize the condition 13 for lectin crystal growth, the phase separation-like crystals obtained from the equilibrated 5 mg/ml of purified lectin under the condition of 18% (w/v) PME 2,000, 0.2 M ammonium sulfate, 0.1 M sodium acetate trihydrate, pH 4.6 after 1 month of incubation at 18°C (Figure 4.15A) and under the condition of 26% (w/v) PME 2,000, 0.2 M ammonium sulfate, 0.1 M sodium acetate trihydrate, pH 4.6 after 1 month of incubation at 18°C (Figure 4.15B). Because of the phase separation-like crystals from these conditions were not stable in cryosolution mother liquor so they could not be mounted for X-ray diffraction analysis.

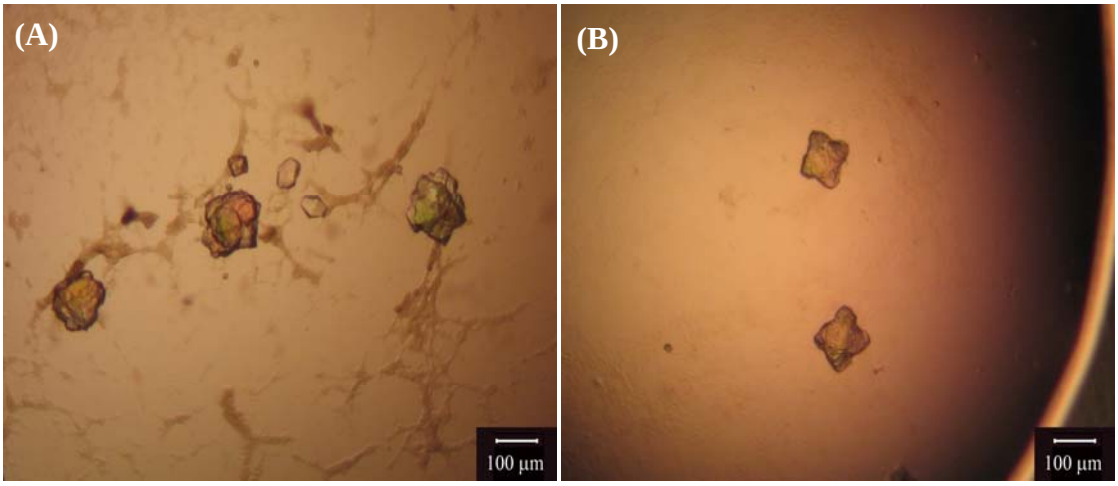


Figure 4.15 Crystals of MC131 lectin grown in hanging drop method using conditions: 18% (w/v) polyethylene glycol monomethyl ether 2,000, 0.2 M ammonium sulfate, 0.1 M sodium acetate trihydrate, pH 4.6 after 1 month of incubation at 18°C (A) and 26% (w/v) polyethylene glycol monomethyl ether 2,000, 0.2 M ammonium sulfate, 0.1 M sodium acetate trihydrate, pH 4.6 after 1 month of incubation at 18°C (B).

For optimization of the condition 22 from Crystal Screen HR2-112; the buffer condition; 12% (w/v) polyethylene glycol 20,000 (PEG 20,000) in 0.1 M MES monohydrate, pH 6.5, were investigated. The concentrations of PEG 20,000, the pH of 0.1 M MES monohydrate buffer, and 0.1 M MOPS, pH 7.0 were studied. A grid of 24 conditions of 0.1 M MES monohydrate, pH 5.5, 6.0 and 6.5, 0.1 M MOPS, pH 7.0, and PEG 20,000 concentrations of 4, 8, 12, 16, 20 and 24% were prepared (Table 4.13).

Table 4.13 Sparse matrix for optimization of the condition 22 for MC131 lectin crystal growth using hanging drop vapor diffusion method.

pH	Concentration of PEG 20,000 (w/v)					
	4%	8%	12%	16%	20%	24%
0.1 M MES, pH 5.5	5.5	5.5	5.5	5.5	5.5	5.5
	4%	8%	12%	16%	20%	24%
0.1 M MES, pH 6.0	6.0	6.0	6.0	6.0	6.0	6.0
	4%	8%	12%	16%	20%	24%
0.1 M MES, pH 6.5	6.5	6.5	6.5	**6.5**	**6.5**	6.5
	4%	8%	12%	**16%**	**20%**	24%
0.1 M MOPS, pH 7.0	7.0	7.0	7.0	7.0	7.0	7.0
	4%	8%	12%	16%	20%	24%

Results from optimization of the lectin crystals under the condition 22, the needle-like crystals obtained from the equilibrated 5 mg/ml of purified lectin after 5 min of incubation at 18°C under the condition; 20% (w/v) polyethylene glycol 20,000 in 0.1 M MES monohydrate, pH 6.5 (Figure 4.16A) whereas the condition; 16% (w/v) PEG 20,000 in 0.1 M MES monohydrate, pH 6.5 gave the plate-like crystals after 2 weaks of incubation (Figure 4.16B).

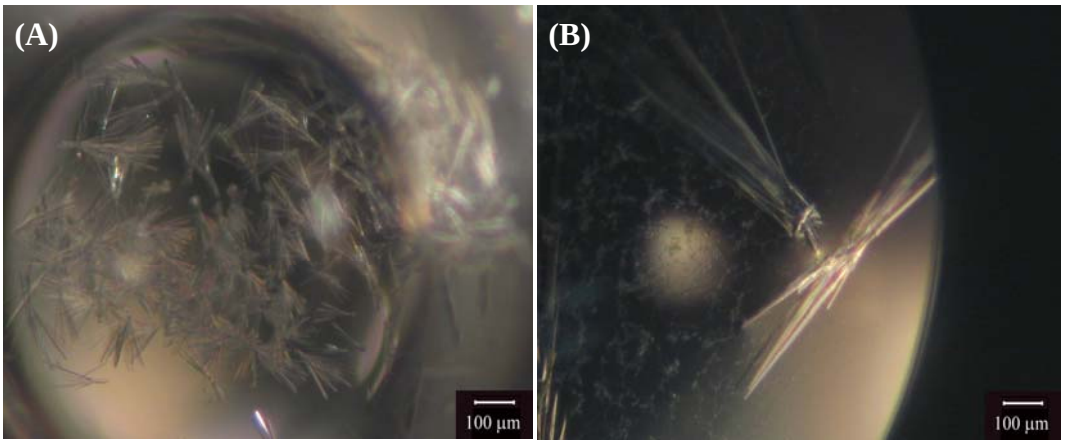


Figure 4.16 Crystals of MC131 lectin grown in hanging drops prepared using conditions: 20% (w/v) polyethylene glycol 20,000 in 0.1 M MES, pH 6.5 after 5 min incubation at 18°C (A), and 16% (w/v) polyethylene glycol 20,000 in 0.1 M MES monohydrate, pH 6.5 after 2 weeks incubation at 18°C (B).

Finally, the plate-like crystals suitable for X-ray diffraction were obtained under the condition; 20% (w/v) polyethylene glycol 20,000 in 0.1 M MES monohydrate, pH 6.5 after two months of incubation at 18°C (Figure 4.17A). Therefore the plate-like crystals suitable for X-ray diffraction were chosen (Figure 4.17B).

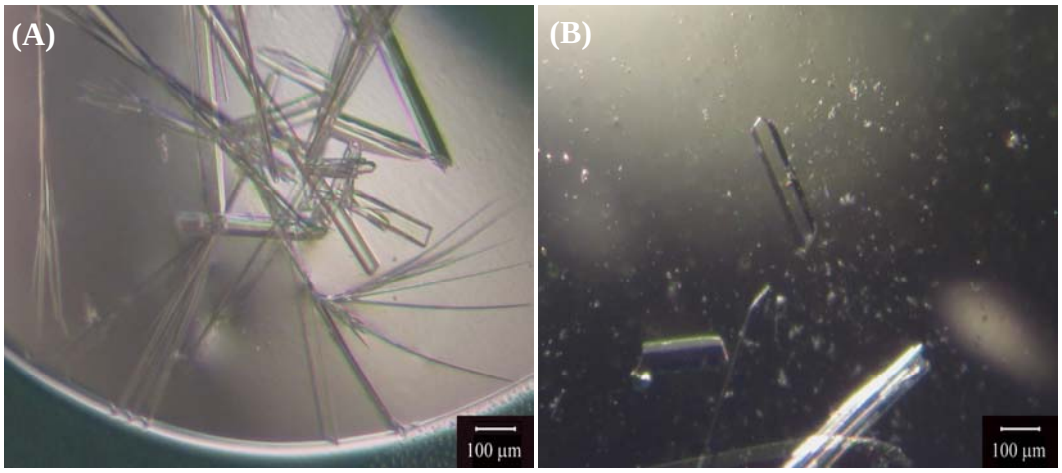


Figure 4.17 The MC131 lectin crystals grown in hanging drop method using the condition: 20% (w/v) polyethylene glycol 20,000 in 0.1 M MES, pH 6.5 after 2 months incubation at 18°C (A), the crystal was chosen for X-ray diffraction (B).

The crystal was subjected to SDS-PAGE analysis. It showed the purified band of MC131 lectin, thus confirming that it was protein crystal (Figure 4.18).

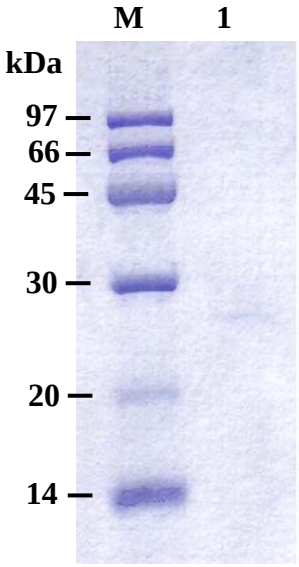


Figure 4.18 SDS-PAGE (12.5% Gel) of the crystal of MC131 lectin from the condition of 20% (w/v) polyethylene glycol 20,000 in 0.1 M MES, pH 6.5 after 2 months incubation at 18°C (lane 1); lane M, low molecular weight protein markers (GE Healthcare).

4.8.2 X-ray diffraction of the selected straw mushroom lectin crystals

After MC131 lectin crystals were successfully grown, X-ray diffraction analysis could be performed to elucidate whether they are macromolecules or salts. 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).

Before data collection, the crystals briefly soaked in cryosolution mother liquor for 2 min containing 15% (v/v) glycerol in the same ingredients as used for crystallization, were further flash-cooled in a nitrogen stream. The X-ray diffraction intensities from MC131 lectin crystals were measured at 100 K at MX end station at the Synchrotron Light Research Institute (Public Organization) (SLRI), facility with a Mar165 CCD detector. The X-ray diffraction data were collected at a wavelength of 1.542 Å. The preliminary X-ray diffraction clearly showed that the crystals of MC131 lectin could diffract to 3.0 Å resolution (Figure 4.19). Analysis of the collected data indicated that MC131 lectin crystals belong to a monoclinic P2 system with unit-cell parameters a = 68.65, b = 80.03 and c = 105.98 Å, α = 90.0°, β = 106.4° and γ = 90.0°. 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. This study was successfully proved that the straw mushroom could provide lectin crystals for structural biological analysis.

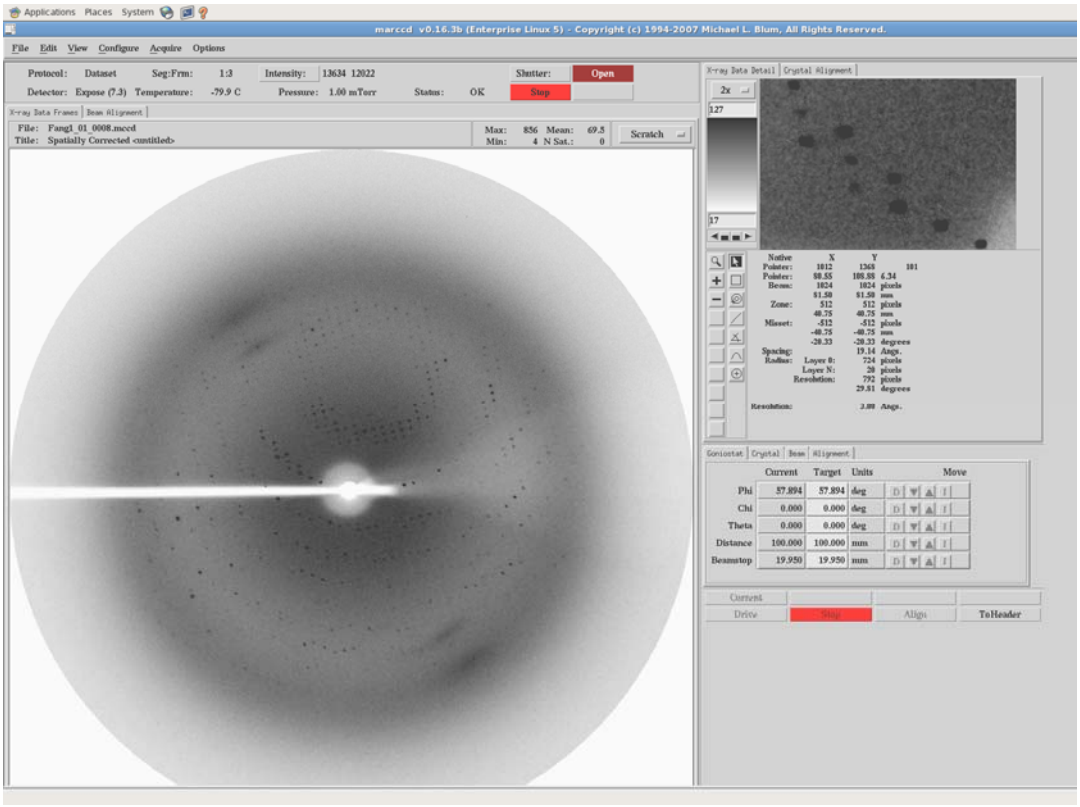


Figure 4.19 X-ray diffraction pattern from a crystal of MC131 lectin.

4.9 Genetic characterization of the selected straw mushroom specimen

The genomic DNA of straw mushroom specimen MC131 was extracted from fruiting body. The genomic DNA was detected in 1% agarose gel electrophoresis. The quality and quantity of the genomic DNA extracted were measured and adjusted to a suitable concentration for PCR amplification. The amplification of 18S rRNA gene was divided into two steps by using two sets of primers (NS1/NS4 primers and SR8R/NS8 primers). The sizes of amplified 18S rRNA gene of straw mushroom MC131 from NS1/NS4 primers and SR8R/NS8 primers were the same size approximately 1,100 bp (Figure 4.20). Both amplified fragments were then combined to obtain the whole 18S rRNA gene sequence.

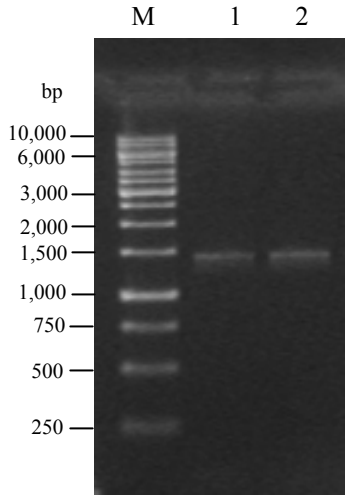


Figure 4.20 Gel electrophoresis of partial 18S rRNA gene fragments of straw mushroom MC131 using different pairs of PCR primers. Lanes: M, DNA marker (GeneRuler 1 kb DNA Ladder, Fermentas); 1, NS1/NS4 primers; 2 SR8R/NS8 primers.

After sequencing, the whole 18S rRNA gene sequences (1,687 bp) of straw mushroom specimen MC131 was compared to those available in GenBank database using standard nucleotide-nucleotide BLAST program to ascertain its closest relatives. The result showed that MC131 was 98% similarity to *Volvariella volvacea* JM leg. SLR (Moncalvo *et al*., 2007), and 97% similarity to *Volvariella volvacea* V23 (Huang *et al*., 2009) and *Volvariella volvacea* strain Vv34 (Huang *et al*., 2009) from GenBank database (Tables 4.14 and 4.15, and Figures 4.21 and 4.22).

Table 4.14 Similarity of 18S rRNA gene sequence of straw mushroom MC131 compared to *Volvariella volvacea* strains from nucleotide sequence database (NCBI).

Mushroom code	**Length of sequence (nt)**	**Nucleotide sequence comparison, identification result**			
		Closest relative	**Length of sequence (bp)**	**Sequence similarity (%)**	**GenBank accession number**
MC131	1,687	*Volvariella volvacea* JM leg. SRL (U.S.A.)	1,713	98	DQ851588
		Volvariella volvacea Vv34 (Chaina)	1,730	97	FJ869178
		Volvariella volvacea V23 (Chaina)	1,718	97	FJ869177

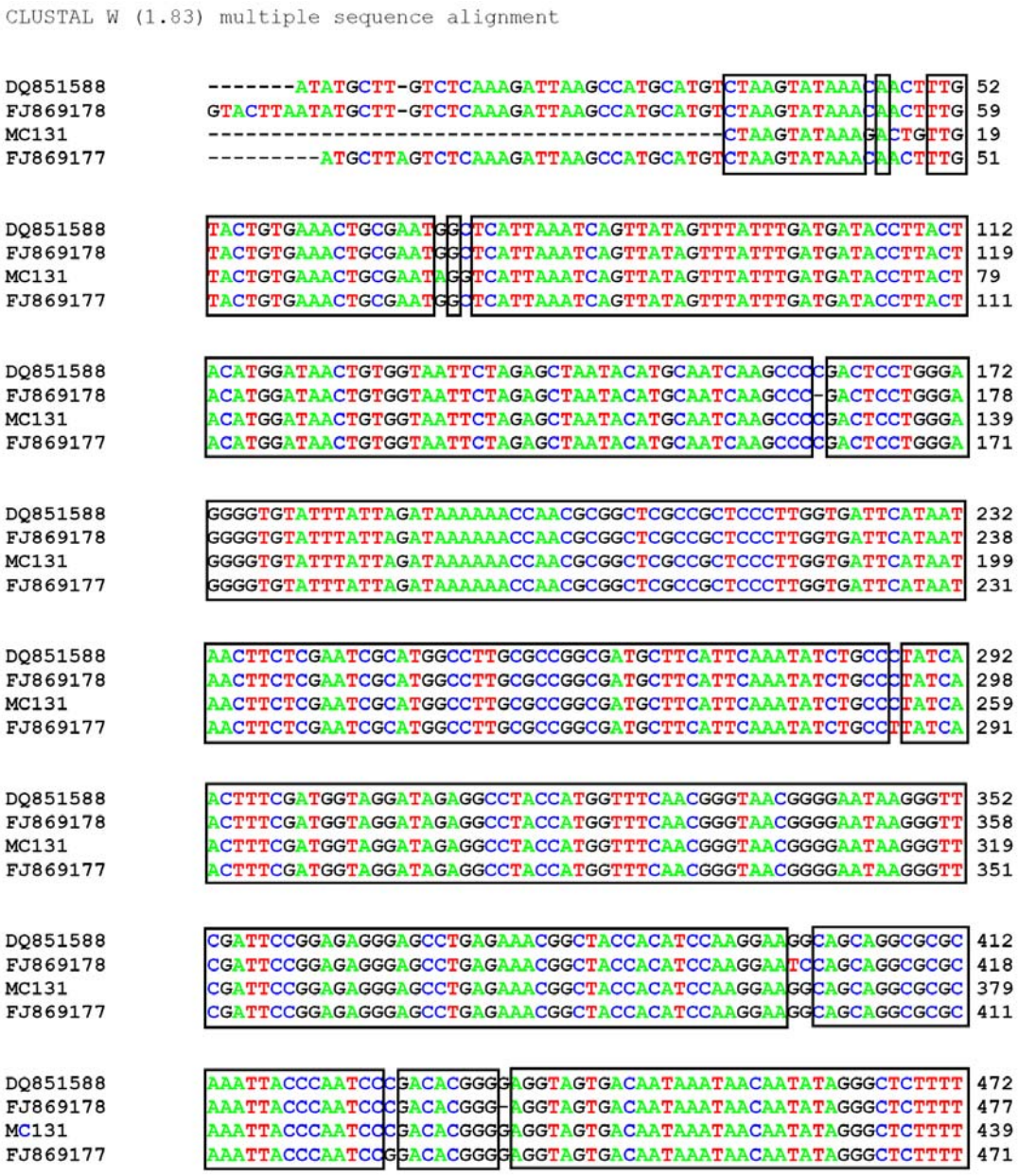


Figure 4.21 Nucleotide sequence alignment of 18S rRNA gene of straw mushroom MC131 and sequences from GenBank database; *Volvariella volvacea* JM leg. SLR (DQ851588), *Volvariella volvacea* V23 (FJ869177) and *Volvariella volvacea* Vv34 (FJ869178) by using ClustalW program. Blocks indicate the conserved nucleotides.

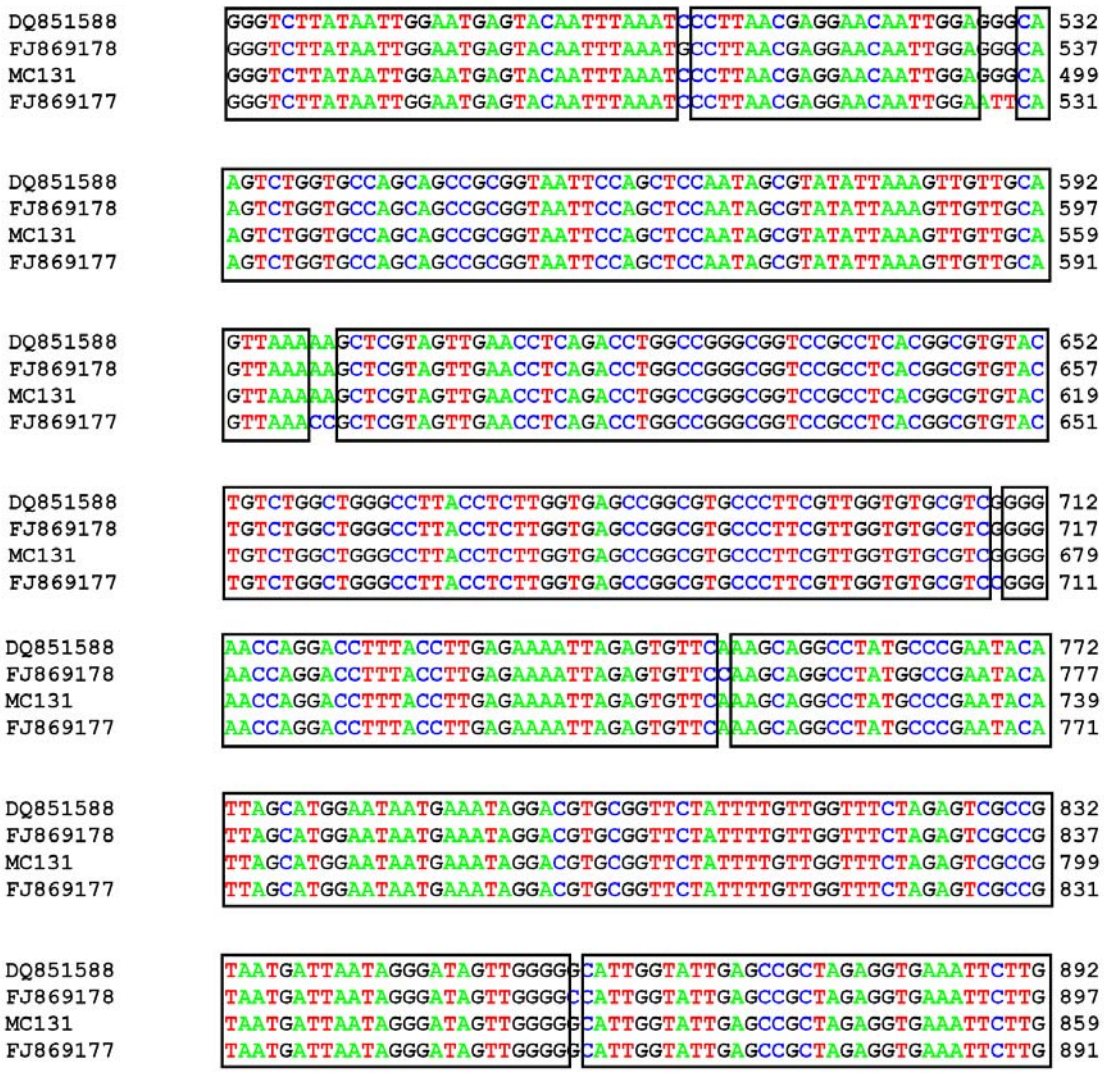


Figure 4.21 (Continued) Nucleotide sequence alignment of 18S rRNA gene of straw mushroom MC131 and sequences from GenBank database; *Volvariella volvacea* JM leg. SLR (DQ851588), *Volvariella volvacea* V23 (FJ869177) and *Volvariella volvacea* Vv34 (FJ869178) by using ClustalW program. Blocks indicate the conserved nucleotides.

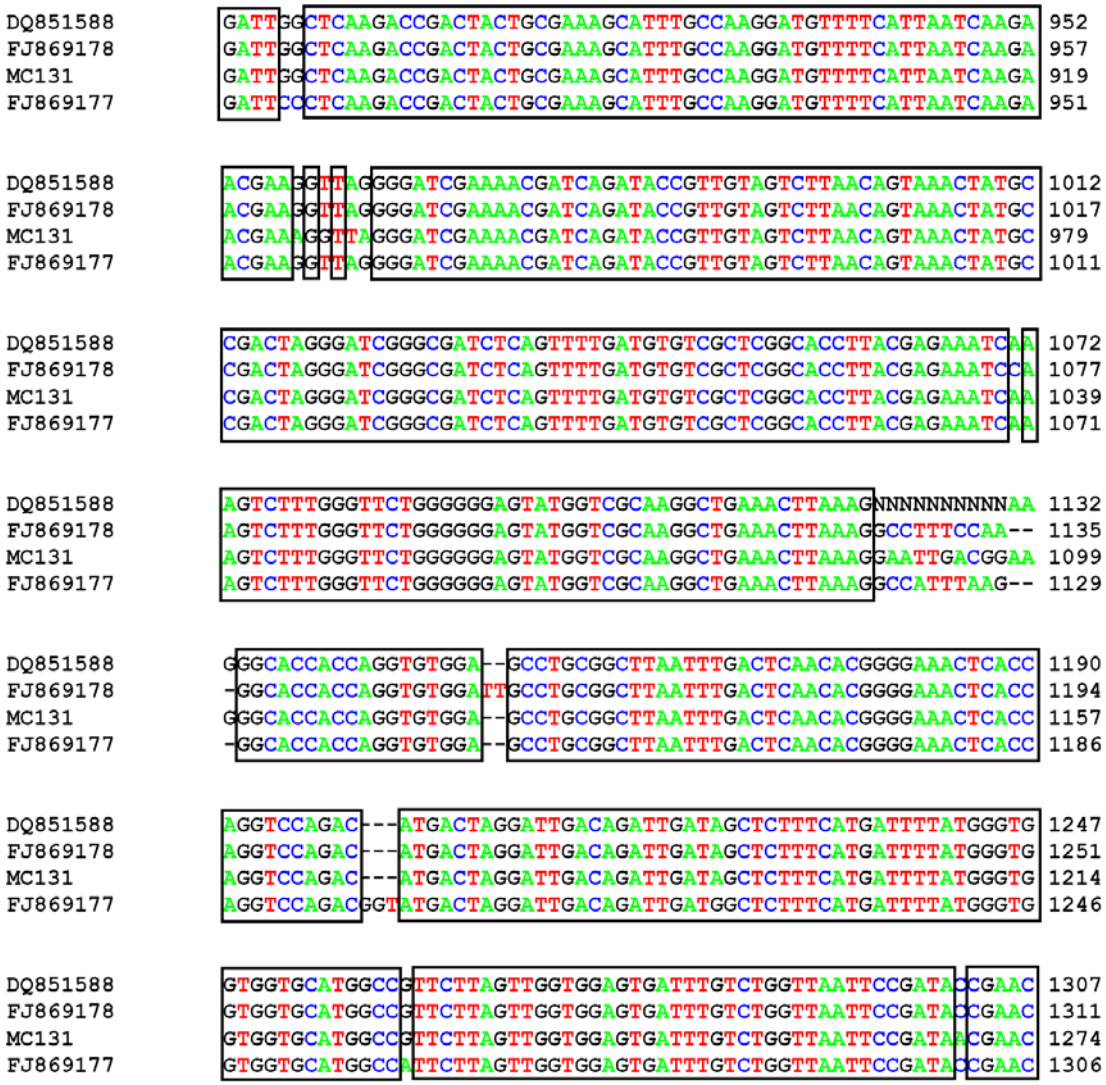


Figure 4.21 (Continued) Nucleotide sequence alignment of 18S rRNA gene of straw mushroom MC131 and sequences from GenBank database; *Volvariella volvacea* JM leg. SLR (DQ851588), *Volvariella volvacea* V23 (FJ869177) and *Volvariella volvacea* Vv34 (FJ869178) by using ClustalW program. Blocks indicate the conserved nucleotides.

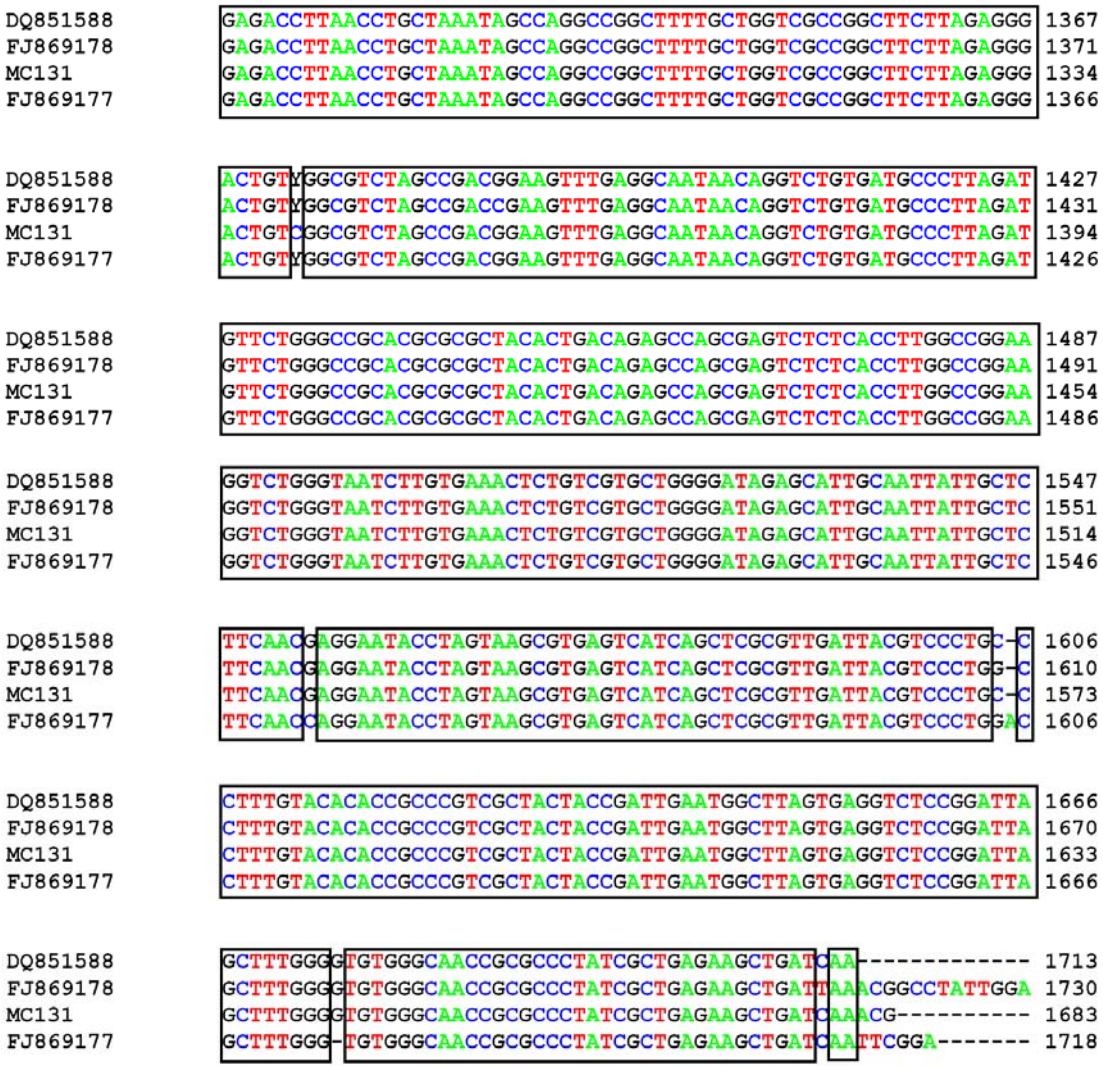


Figure 4.21 (Continued) Nucleotide sequence alignment of 18S rRNA gene of straw mushroom MC131 and sequences from GenBank database; *Volvariella volvacea* JM leg. SLR (DQ851588), *Volvariella volvacea* V23 (FJ869177) and *Volvariella volvacea* Vv34 (FJ869178) by using ClustalW program. Blocks indicate the conserved nucleotides.

Table 4.15 Comparison of sequence similarity of 18S rRNA gene of straw mushroom MC131 to *Volvariella volvacea* strains from GenBank database, U.S.A. and closest relatives

Mushroom species	MC131	1	2	3	4	5	6	7	8	9	10	11	12	13
MC131	100													
1	98	100												
2	98	98	100											
3	98	98	97	100										
4	98	98	98	98	100									
5	98	98	98	98	98	100								
6	98	98	98	98	98	98	100							
7	98	98	98	98	98	98	98	100						
8	98	98	98	98	98	96	98	98	100					
9	98	98	98	98	98	98	98	98	98	100				
10	98	98	98	98	98	98	96	98	98	98	100			
11	98	98	98	98	98	98	97	98	98	98	98	100		
12	98	98	98	98	98	98	98	98	98	98	97	98	100	
13	98	98	98	98	98	98	98	98	98	98	98	98	98	100

MC131:, 1: *Volvariella volvacea* Vv34 (FJ869178), 2: *Volvariella volvacea* V23 (FJ869177), 3: *Volvariella volvacea* JM leg. SRL (DQ851588), 4: *Hohenbuehelia tristis* RV95/214 (DQ851573), 5: *Xeromphalina campanella* isolate AFTOL-ID 1524 (DQ465344), 6: *Agrocybe erebia* isolate AFTOL-ID 1807 (DQ440631), 7: *Tricholoma myomyces* strain KMS589 (DQ367422), 8: *Coprinopsis atramentaria* isolate AFTOL-ID 1496 (DQ115781), 9: *Mythicomyces corneipes* isolate AFTOL-ID 972 (DQ092917), 10: *Tricholoma aestuans* isolate AFTOL-ID 497 (AY757267), 11: *Coprinus comatus* isolate AFTOL-ID 626(AY665772), 12: *Tricholoma saponaceum* isolate AFTOL-ID 672 (AY654883), 13: *Termitomyces* sp. (AB051891).

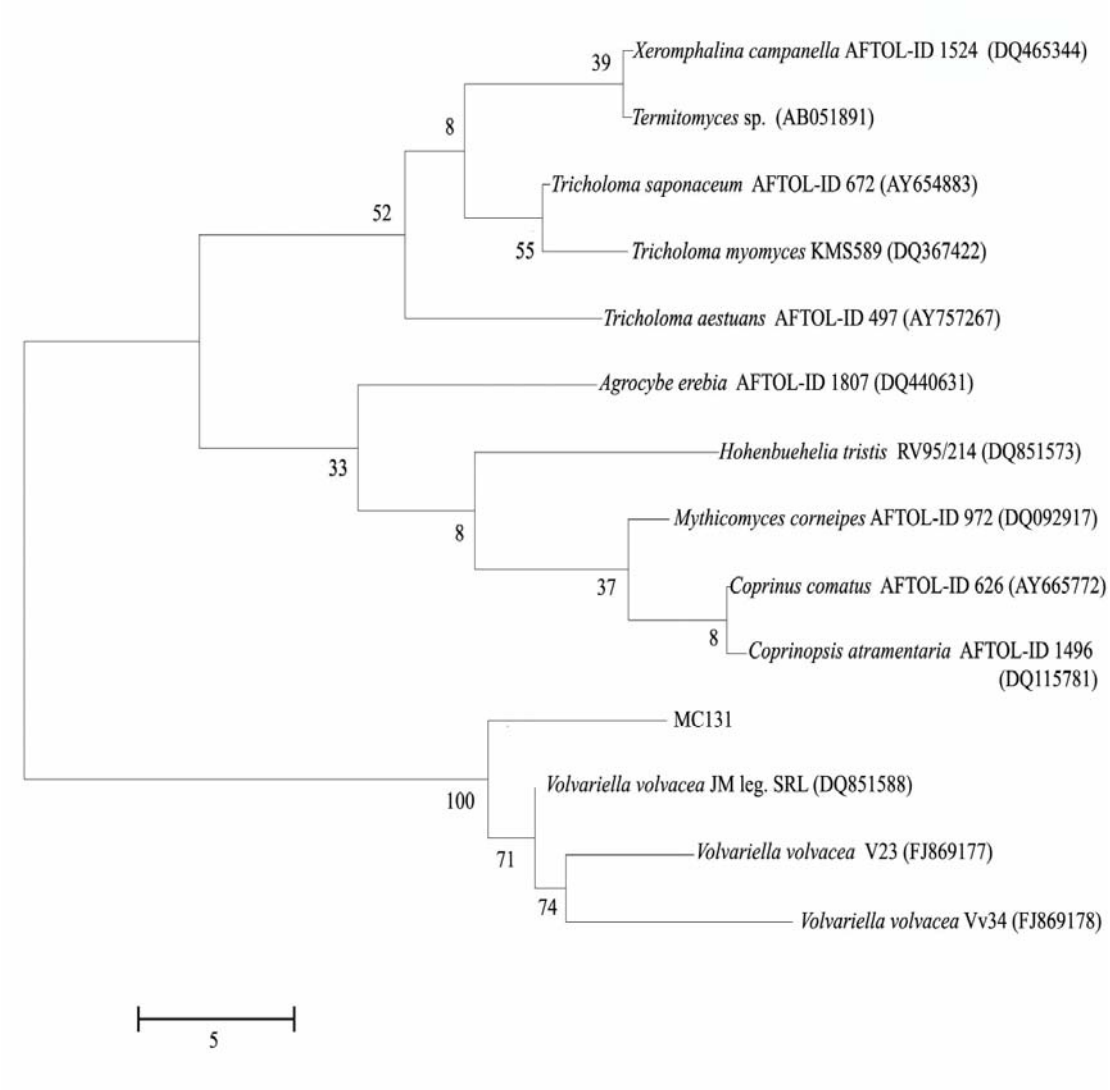


Figure 4.22 Phylogenetic tree of straw mushroom MC131 based on 18S rRNA gene sequence data using the neighbor-joining method. The unrooted tree was derived by using BioEdit and MEGA version 4.0 programs. Branch lengths are scaled in terms of expected numbers of nucleotide substitution per site. Numbers on branches are bootstrap values from 1,000 replications. The scale bar represents (calculated) distance.

CHAPTER V

CONCLUSION

In this study, a total of 11 straw mushroom specimens (fruiting bodies): MC131-134, MC138-140, MC144-145 and MC167-168, were collected from local markets in 9 provinces: Nakhon Ratchasima, Surin, Buriram, Khon Kaen, Kalasin, Chaiyaphum, Maha Sarakham, Sakon Nakhon and Ubon Ratchathani Provinces in North-eastern Thailand. The mushrooms were cultivated in only 6 provinces which were Nakhon Ratchasima, Surin, Buriram, Khon Kaen, Kalasin and Sisaket Provinces. The specimens were, then, identified using their morphological characteristics. All 11 specimens belonged to *Volvariella volvacea*. The collected fruiting bodies were separated into parts of fruiting body: cap and stalk, as well as whole fruiting body and preserved for lectin accumulation by drying at 40°C for approximately 12 h. Crude lectins were extracted from cap, stalk and whole fruiting body by grinding the dried fruiting bodies into powder and adding PBS (10 mM phosphate buffer saline, pH 7.4 containing 2 mM sodium bisulphite) to make up dilution of 1:10 (w/v), and detected by hemagglutination assay using rabbit red blood cells. Cap, stalk and whole fruiting body extracts of all 11 specimens displayed hemagglutination titers on the average of 2,048, 2,048 and 4,096 respectively. The crude lectin extracts were then tested for their antimicrobial and cytotoxic activities in order to select a mushroom specimen for further purification and characterization of its lectin.

From antimicrobial activity test using the agar disc diffusion method, only four lectin extracts: MC131, MC133, MC134 and MC145; and two extracts: MC145 and MC168, showed antibacterial and antifungal activities respectively. Crude lectins from straw mushroom specimen codes MC131 and MC133 could inhibit *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922 at concentrations of 0.090 and 0.123, 0.057 and 0.053, and 0.116 and 0.128 μg/μl, respectively, compared to standard streptomycin. Extracts of MC134 and MC145 inhibited the growth of *S. aureus* ATCC 29213 at concentrations of 0.066 and 0.065 μg/μl, respectively. MC145 and MC168 extracts could inhibit *Aspergillus niger* ATCC 6275 at 1.65 and 1.46 times, respectively, narrower than standard nystatin (100 units) inhibition zone diameter. MC168 extract also inhibited the growth of *Candida albican* ATCC 10231 and *Penicillium funiculosum* ATCC 36839 at 0.60 times and 1.38 times narrower and wider than standard nystatin inhibition zone, respectively

These crude lectin extracts were then tested for cytotoxicity against two types of human cancer cells: human epidermoid carcinoma (KB) and human cervical carcinoma (HeLa) cells. The cytotoxicity toward KB cells was observed on crude extracts of MC131, MC133, MC134, MC138 and MC167 with IC_{50} values of 4.80, 1.40, 4.06, 5.08 and 7.30 μg/ml respectively. Only MC134 extract, showed cytotoxicity against HeLa cell with IC_{50} value of 21.38 μg/ml.

The straw mushroom specimen MC131 obtained from Phimai District, Nakhon Ratchasima Province, was found to accumulate the same titers (1024) of lectin in its cap, stalk and whole fruiting body which was higher than lectins accumulated in fruiting body of some other specimens collected when tested against rabbit red blood cells. Crude MC131 lectins also exhibited both antibacterial and

cytotoxicity activities. Therefore, MC131 was selected for its lectin purification and characterization. For purification of straw mushroom lectins, three steps: 90% $(NH_4)_2SO_4$ precipitation, anion exchange chromatography on DEAE-sepharose column and gel filtration by fast protein liquid chromatography on Superdex 75 column, were found to be suitable. From specific activity of crude lectin extract of MC131 at 2.50 units/mg, the specific activity of the lectin was increased up to 123.31 units/mg which were 49.27 folds after 3 purification steps. The concentration of purified lectin (9.34 mg) could be obtained from 100 g of dried straw mushroom powder.

The purified MC131 lectin had its molecular weight of 25 kDa characterized by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing condition. Its pI determined by 2D-electrophoresis was about 5. The MC131 lectin was glycoprotein, which was not specific to 32 sugars tested; *N*-acetyl-D-galactosamine (GalNAc), 2-acetamido-2-deoxy-D-glucopyranose, D-adonitol, amygdalin, L-arabinose, cellobiose, esculin, fructose, fucose, D-galactose, D-glucose, inositol, inulin, lactose, lactitol, maltose, mannitol, D-mannose, D-melezitose, melibiose, methyl-α-D-galactopyranoside, methyl-β-D-galactopyranoside, 4-nitrophynyl-α-D-galactopyranoside, 4-nitrophynyl-β-D-galactopyranoside, raffinose, rhamnose, ribose, salicin, sorbitol, trehalose, D-turanose and D-xylose. Amino acid sequence of the purified 25 kDa protein showed 99% similarity to the 24.2 kDa protein of volvatoxin A2 precursor of *Volvariella volvacea* (AAQ92757.1) from GenBank database U.S.A. The lectin was stable at temperature up to 60°C for 30 min and at pH 7 for 18 h test whereas crude lectin extract of MC131 was stable at temperature up to 60°C for 1 h and at pH 5-12 for 18 h test. The lectin did not exhibit antimicrobial activity as the

crude extract had, but it showed cytotoxicity against KB and HeLa cells with IC_{50} values of 0.58 and 0.70 μg/ml respectively.

Purified MC131 lectin at concentration of 25 mg/ml was used for screening crystallization conditions using the microbatch under oil technique with Hampton Research Crystal Screen HR2-110 and HR2-112 (Hampton Research, California, U.S.A.) at 18°C. Six out of 98 conditions provided needle-like and plate-like crystals within 1 and 3 days of incubation. These conditions were then further investigated using hanging drop vapor diffusion method. After a week of incubation, three out of 6 conditions gave needle-like and plate-like crystals. The 3 conditions were, therefore, selected for optimization of lectin crystal growth conditions focused on protein concentration, precipitant concentration and pH of crystallization solution. The MC131 lectin crystal growth was successfully obtained with conditions: 5 mg/ml of lectin in the buffer composed of 20% (w/v) polyethylene glycol (PEG) 20,000 in 0.1 M MES, pH 6.5 with incubated at 18°C for 2 months. This condition gave plate-like crystals. The MC131 lectin crystal could diffract to 3.0 Å resolution, which performed as X-ray diffraction experiments, which were carried out at 100 K using a Microstar™ rotating-anode X-ray generator at the Synchrotron Light Research Institute (SLRI), Thailand, and a Mar165 charge-coupled device (CCD) detector on the Macromolecule Crystallography end station.

Since each straw mushroom specimen collected in this study accumulated different concentrations of lectins with different biological properties, strain characterization of MC131 was performed. MC131 had its 18S rRNA gene sequence (1,687 bp) 98% similarity to *Volvariella volvacea* JM leg. SLR (Moncalvo *et al*.,

2007), 97% similarity to *Volvariella volvacea* Vv34 (Huang *et al*., 2009) and *Volvariella volvacea* V23 (Huang *et al*., 2009) from GenBank database U.S.A.

This study was successfully proved that lectins from straw mushroom cultivated in different locations in North-eastern Thailand exhibited different biological properties, antimicrobial activity and cytotoxicity, that will be useful for further applications. The single crystal of purified straw mushroom lectin obtained could be beneficial for studying three dimensional structure.

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APPENDICES

APPENDIX A

REAGENT AND CULTURE MEDIUM PREPARATIONS

1. Buffers 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 bisulphite (0.02 M)	22.80	g

The ingredients were dissolved and adjusted pH to 7.4 with HCl. Then the final volume was adjusted to 1,000 ml with deionized water.

1.2 PBS for the extraction in the antimicrobial assay and purification

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

Polyvinylpyrrolidone (PVPP) (1.5%)	7.50	g
NaN_3 (0.02%)	0.20	g

The components were dissolved and adjusted pH to 7.4 with HCl. Then the final volume was adjusted to 1,000 ml with deionized water.

2. Buffers and solutions for mushroom lectin purification

2.1 Running buffer for the ion-exchange chromatography (DEAE-Sepharose)

Tris (10 mM, prepared from 2M stock solution Tris-HCl, pH 8)	5.00	ml

The solution was dissolved and adjusted pH to 7.4 with HCl. Then the final volume was adjusted to 1,000 ml with deionized water.

2.2 Tris (2 M, pH 8.0)

Tris (2 M)	242.28	g

The chemical was dissolved and adjusted pH to 8.0 with HCl. Then the final volume was adjusted to 1,000 ml with deionized water.

2.3 Running buffer for gel filtration chromatography

Tris (10 mM, prepared from 2 M stock solution Tris-HCl, pH 8)	5.00	ml
NaCl (100 mM)	0.0058	g

The components were dissolved and adjusted pH to 7.4 with HCl. Then the final volume was adjusted to 1,000 ml with deionized water.

3. Buffers and solutions for SDS-PAGE

3.1 Resolving gel SDS-PAGE (12.5%)

Tris-HCl (1.5 M, pH 8.8)	3.75	ml
Bis-acrylamide (1%)	1.12	ml
Deionized water	1.14	ml
Sodium dodecyl sulfate (SDS) (20%)	0.15	ml
Acrylamide (30%)	8.75	ml
Ammonium persulfate (10%)	0.10	g
N,N,N′,N′-Tetramethylethylenediamine (TEMED)	0.01	ml

3.2 Stacking gel SDS-PAGE (5%)

Tris-HCl (1 M, pH 6.8)	3.75	ml
Bis-acrylamide	3.90	ml
Deionized water	4.00	ml
Sodium dodecyl sulfate (SDS) (20%)	0.15	ml
Acrylamide (30%)	2.50	ml
Bromophenol blue (0.1%)	0.60	ml
Ammonium persulfate (10%)	100.00	µl
N,N,N′,N′-Tetramethylethylenediamine (TEMED)	20.00	µl

3.3 Running buffer for SDS-PAGE

Glycine	14.40	g
Tris-base	3.03	g
Sodium dodecyl sulfate (SDS)	0.50	g

The ingredients were dissolved and adjusted the volume to 1,000 ml with deionized water.

3.4 SDS-gel loading buffer

Tris-HCl (1 M, pH 6.8)	4.00	ml
Sodium dodecyl sulfate (SDS)	1.00	g
2-Mercaptoethanol	0.50	ml
Bromophenol blue (0.1%)	1.00	g
Glycerol	10.00	ml

The components were dissolved and adjusted the volume to 1,000 ml with deionized water.

3.5 Staining solution with coomassie brilliant blue

Coomassie brilliant blue R-250	2.00	g
Methanol	450.00	ml
Glacial acetic acid	100.00	ml

The components were dissolved and adjusted the volume to 1,000 ml with deionized water.

3.6 Destaining solution for coomassie stain

Methanol	450.00	ml
Glacial acetic acid	100.00	ml

The ingredients were dissolved and adjusted the volume to 1,000 ml with deionized water.

3.7 Tris-HCl (1 M, pH 6.8)

Tris-HCl (1 M)	121.14	g

The chemical was dissolved and adjusted pH to 6.8 with HCl. Then the final volume was adjusted to 1,000 ml with deionized water.

4. Chemicals and reagents of periodic aaid Schiff (PAS) staining for detecting gycoprotein

4.1 Fixation

Tricarboxylic acid (TCA) (12.5%)	12.50	ml

The solution was suspended in deionized water, and then adjusted the volume to 100 ml with deionized water.

4.2 Acetic acid (3%)

Acetic acid	3.00	ml

The solution was suspended in deionized water, and then adjusted the volume to 100 ml with deionized water.

4.3 Periodic acid (1%) in acetic acid (3%)

Periodic acid	1.00	g

The chemical was dissolved in 100 ml of 3% acetic acid.

4.4 Potassium meta-bisulfite solution (0.5%)

Potassium meta-bisulphite ($K_2S_2O_5$)	0.50	g

The chemical was dissolve in 100 ml of 3% (v/v) acetic acid (made fresh).

4.5 Fuchsin-sulphite solution

Basic fuchsin	2.00	g
Deionized water	400.00	ml
HCl (2 N)	10.00	ml
Potassium meta-bisulphite ($K_2S_2O_5$)	4.00	g

Two gram of basic fuchsin was dissolved in 400 ml of deionized water with warming the solution, cooled, and then filtrated through Whatman No.1 paper. The solution was added wilth 10 ml of 2 N HCl and dissolved 4 g of $K_2S_2O_5$ in it. Then the solution was placed in a stoppered bottle and kept cool overnight. Next day, the solution was stirred with 1 g of activated charcol, filtrated through Whatman No.1 paper, and then added the sufficient 2 N HCl (10 ml or more) until a drop dried on a galss slide DID NOT turn red. The solution was stored in a cool in the dark place.

4.6 Acetic acid (7%)

Acetic acid	7.00	ml

The solution was suspended in deionized water, and then adjusted the volume to 100 ml with deionized water.

5. Reagents for Bradford protein assay

5.1 Bradford reagent

Coomassie brilliant blue G-250	100.00	mg
Ethanol (95%)	50.00	ml
Phosphoric acid (85% (w/v))	100.00	ml

One hundred milligrams of coomassie brilliant blue G-250 were dissolved in 50 ml of 95% ethanol then added 100 ml of 85% (w/v) phosphoric acid and adjusted the volume to 1,000 ml with deionized water. The Bradford dye was filtrated through Whatman No.1 paper then placed in a stoppered bottle and kept cool in the dark place.

5.2 NaOH (1 M)

NaOH	4.00	g

The chemical was dissolved in 100 ml of deionized water.

6. Chemicals and reagents for genetic characterization

6.1 Lysis buffer

Sodium dodecyl sulfate (SDS) (25%)	25	g
Ethylenediaminetetraacetic acid (EDTA) (50 mM)	1.86	g
NaCl (75 mM)	0.44	g
Tris-HCl (50 mM, pH 7.5)	0.61	g

The ingredients were dissolved and adjusted the volume to 100 ml with deionized water. Then, the solution was sterilized by autoclaving for 10 min at 121°C, 15 lb/square inches after preparation.

6.2 Tris-EDTA (TE) Buffer

Tris Base	1.21	g
Ethylenediaminetetraacetic acid (EDTA)	0.37	g

The ingredients were dissolved and adjusted the volume to 1,000 ml with deionized water. Then, the solution was sterilized by autoclaving for 10 min at 121°C, 15 lb/square inches after preparation.

6.3 Sodium acetate (3 M)

Sodium acetate (CH_3COONa)	24.61	g

The chemical was dissolved in deionized water, adjusted to pH 5.2 with glacial acetic acid, and adjusted the volume to 100 ml with deionized water. Then, the solution was sterilized by autoclaving for 10 min at 121°C, 15 lb/square inches after preparation.

6.4 RNAase (10 mg/ml)

RNAase	10.00	mg

The RNAase was dissolved in 10 mM Tris-HCl (pH 7.5), 15 mM NaCl and stored at -20°C.

6.5 Tris-borate (TBE) buffer (5X)

Tris Base	54.00	g
Boric acid	27.50	g
Ethylenediaminetetraacetic acid (EDTA)	0.37	g

The ingredients were dissolved and adjusted the volume to 1,000 ml with deionized water.

6.6 Gel loading buffer (6X)

Bromophenol blue	25.00	g

The dye was dissolved and adjusted the volume to 10 ml with 40% sucrose in water.

6.7 Ethidium bromide (10 mg/ml)

Ethidium bromide (Sigma)	1.00	g

The chemical was dissolved and adjusted the volume to 10 ml with sterilized deionized water.

7. Reagent for mushroom identification

Lactophenol

Lactic acid	20.00	ml
Phenol crystal	20.00	g
Glycerol	40.00	ml
Deionized water	20.00	ml

The ingredients were dissolved and kept in the bottle (may added 0.05 g of cotton blue or methylene blue).

8. Culture media for antimicrobial activity test

8.1 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 dissolved in deionized water, adjusted to pH 5.4 with HCl, and adjusted the volume to 1000 ml with deionized water. Then, the solution was sterilized by autoclaving for 10 min at 121°C, 15 lb/square inches after preparation.

8.2 Mueller-Hinton agar

Beef, dehydrated infusion form	30.00	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 dissolved in deionized water, adjusted to pH 7.3 with HCl, and adjusted the volume to 1000 ml with deionized water. Then, the solution was sterilized by autoclaving for 10 min at 121°C, 15 lb/square inches after preparation.

8.3 Nutrient broth

Beef extract	3 .00	g
Peptone	5.00	g

Final pH 7.4 ± 0.2 at room temperature

The compositions were dissolved in deionized water, adjusted to pH 7.4 with HCl, and adjusted the volume to 1000 ml with deionized water. Then, the solution

was sterilized by autoclaving for 10 min at 121°C, 15 lb/square inches after preparation.

APPENDIX B

STANDARD CURVES AND AMMONIUM SULPHATE PRECIPITATION TABLE

Different concentrations of bovine serum albumin (BSA) were prepared (Table 1B) for standard curve of Bradford protein assay according to Bradford (1976).

Table 1B The concentration of BSA in the stock solution was 0.2 mg/ml.

Amount of BSA (mg)	Volume of stock BSA solution (µl)	volume of buffer (µl)
0.02	20	80
0.08	40	60
0.12	60	40
0.16	80	20
0.20	100	0

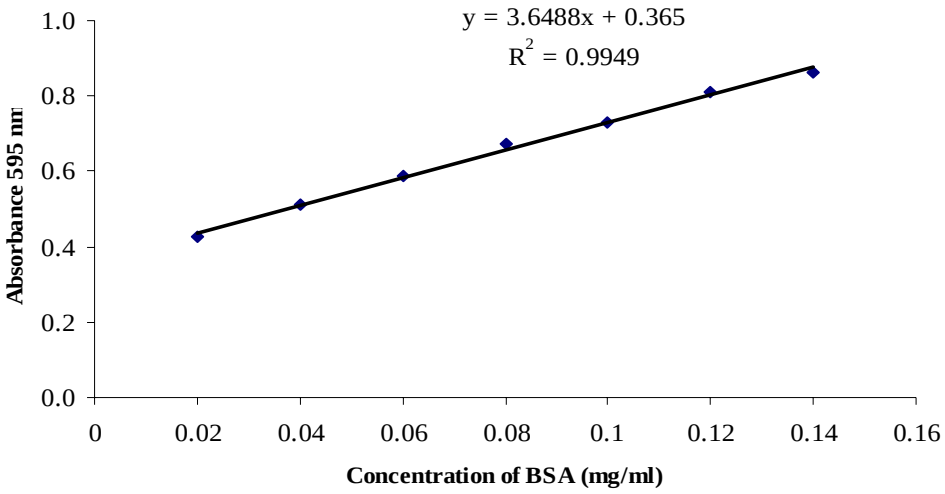

Figure 1B Standard curve of bovine serum albumin (BSA) according to Bradford (1976).

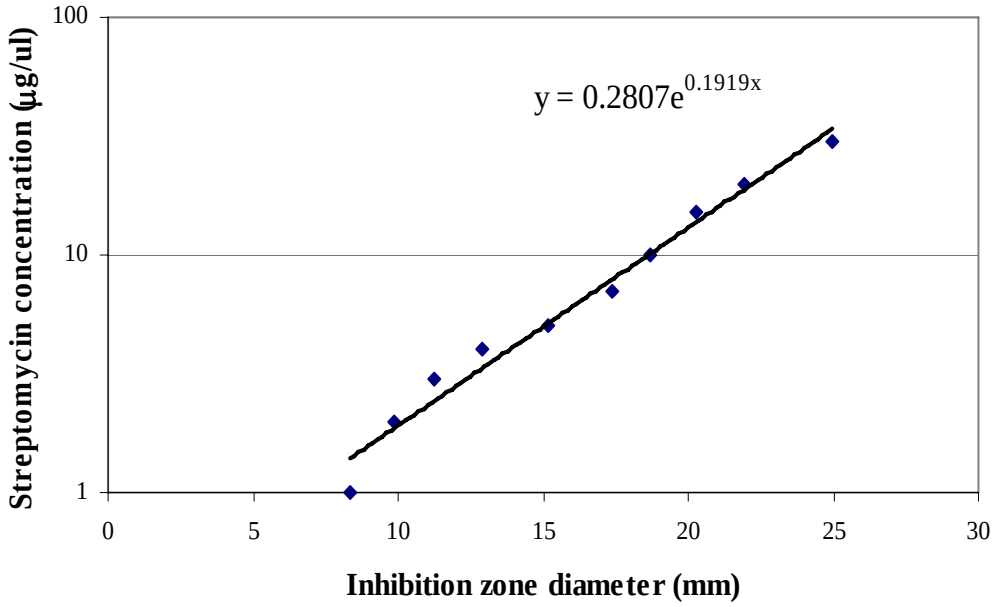

Figure 2B Stand curve of streptomycin tested against *Escherichia coli* ATCC 25922.

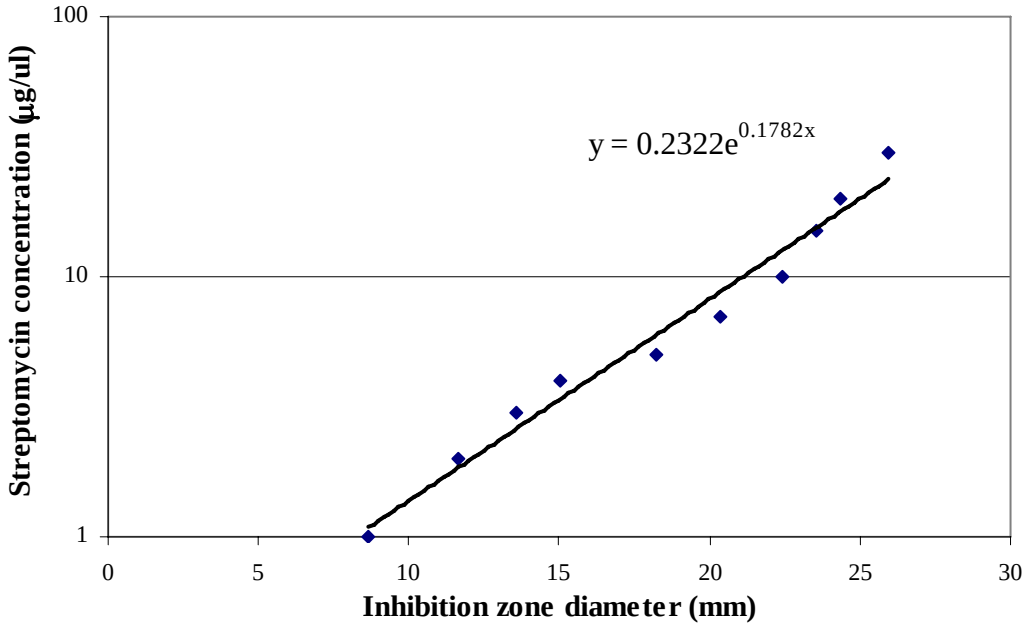

Figure 3B Stand curve of streptomycin tested against *Bacillus subtilis* ATCC 6633.

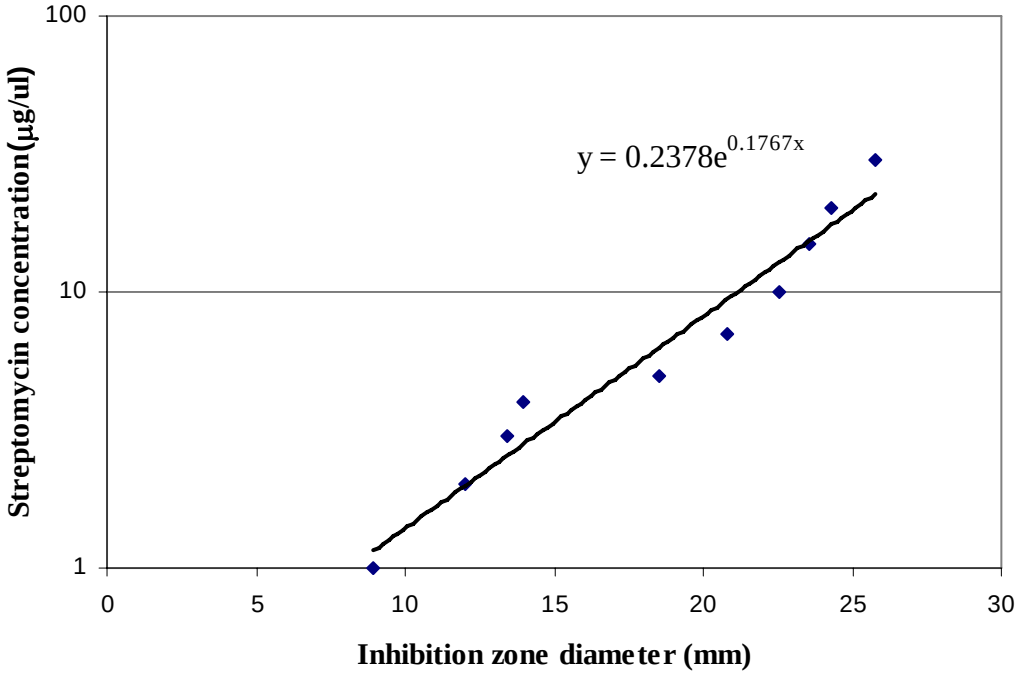

Figure 4B Stand curve of streptomycin tested against *Bacillus cereus* ATCC 11778.

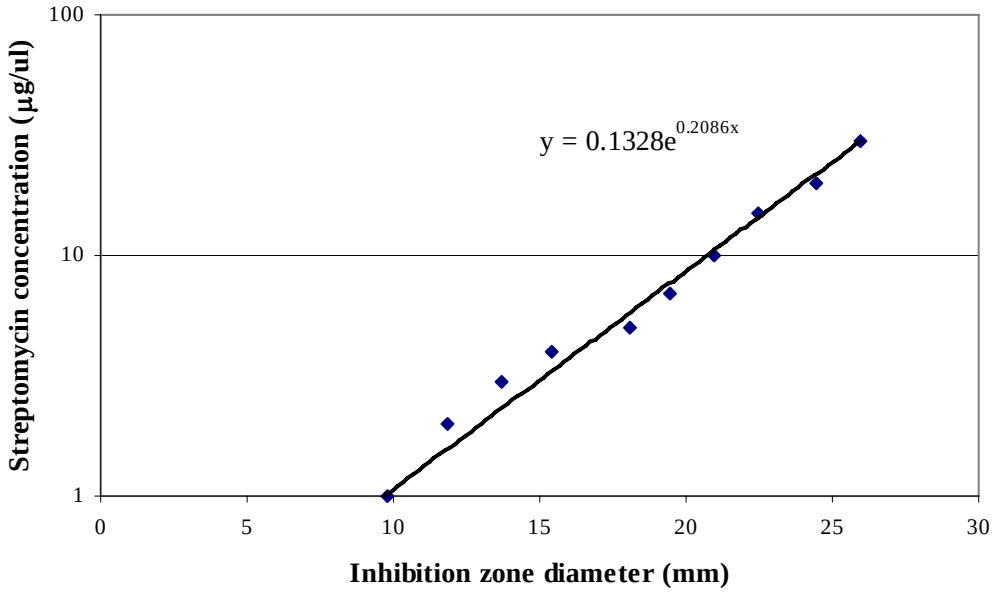

Figure 5B Stand curve of streptomycin tested against *Staphylococcus aureus* ATCC 29213.

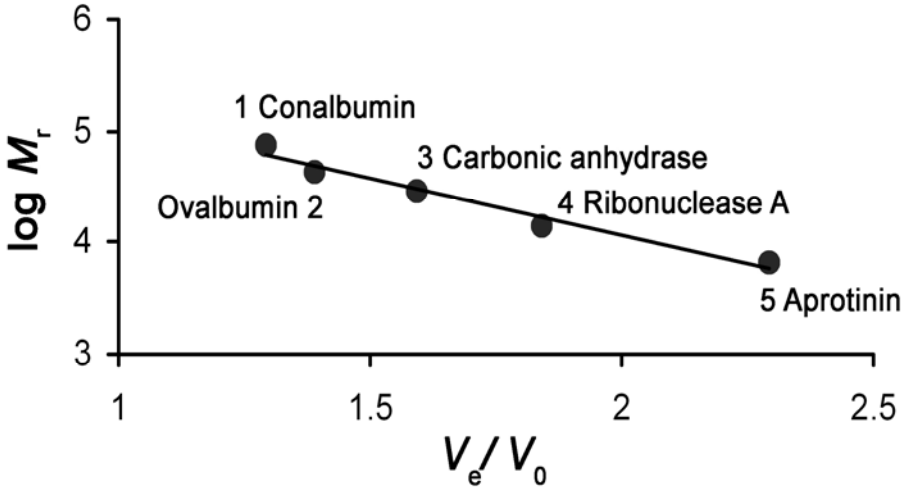

Figure 6B Molecular weight of lectin determined by the calibration curve obtained from gel filtration chromatography.

Table 2B Ammonium sulphate precipitation.

The initial and final concentrations of ammonium sulphate, expressed as percent saturation, are on the vertical and horizontal scales, respectively. The point of intersection of two lines drawn from these points indicates the number of grams of salt to be added to each littler of solution at the initial concentration to lead to the final concentration required.

Initial concentration of ammonium sulphate	Solid ammonium sulphate (grams) to be added to 1 liter of solution															
	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 C

HEMAGGLUTINATION TESTS OF STRAW MUSHROOM EXTRACTS AGAINST RABBIT RED BLOOD CELLS

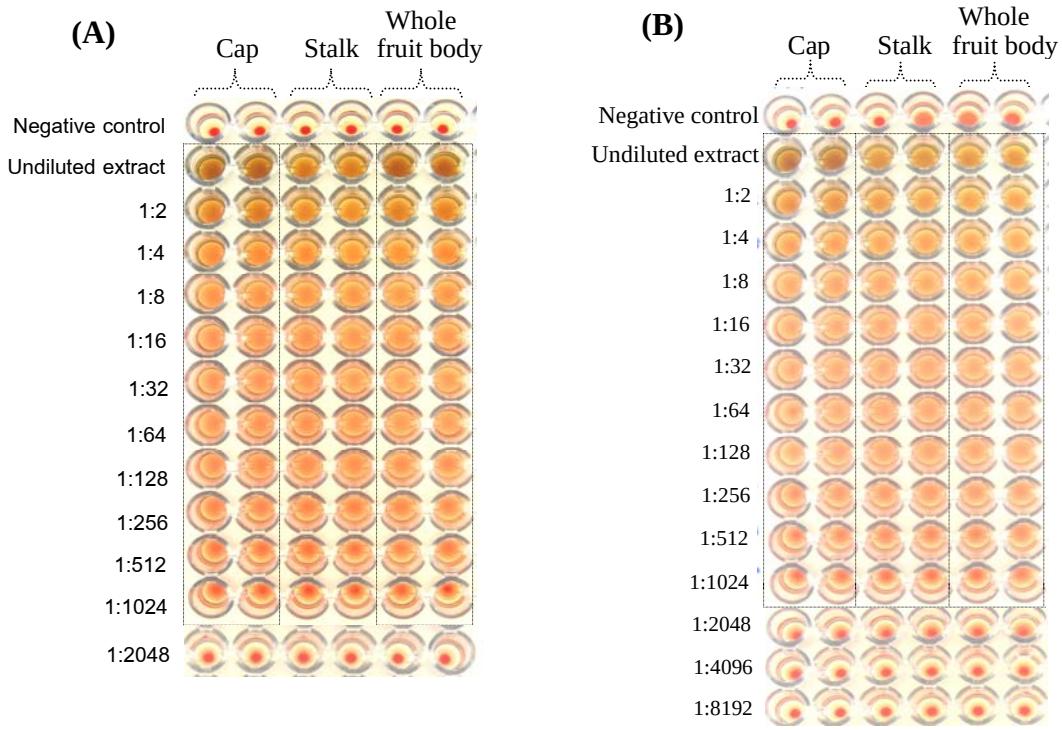

Figure 1C Hemagglutination tests of cap, stalk and whole fruiting body of straw mushroom extracts MC131 (A) and MC132 (B) against rabbit red blood cells.

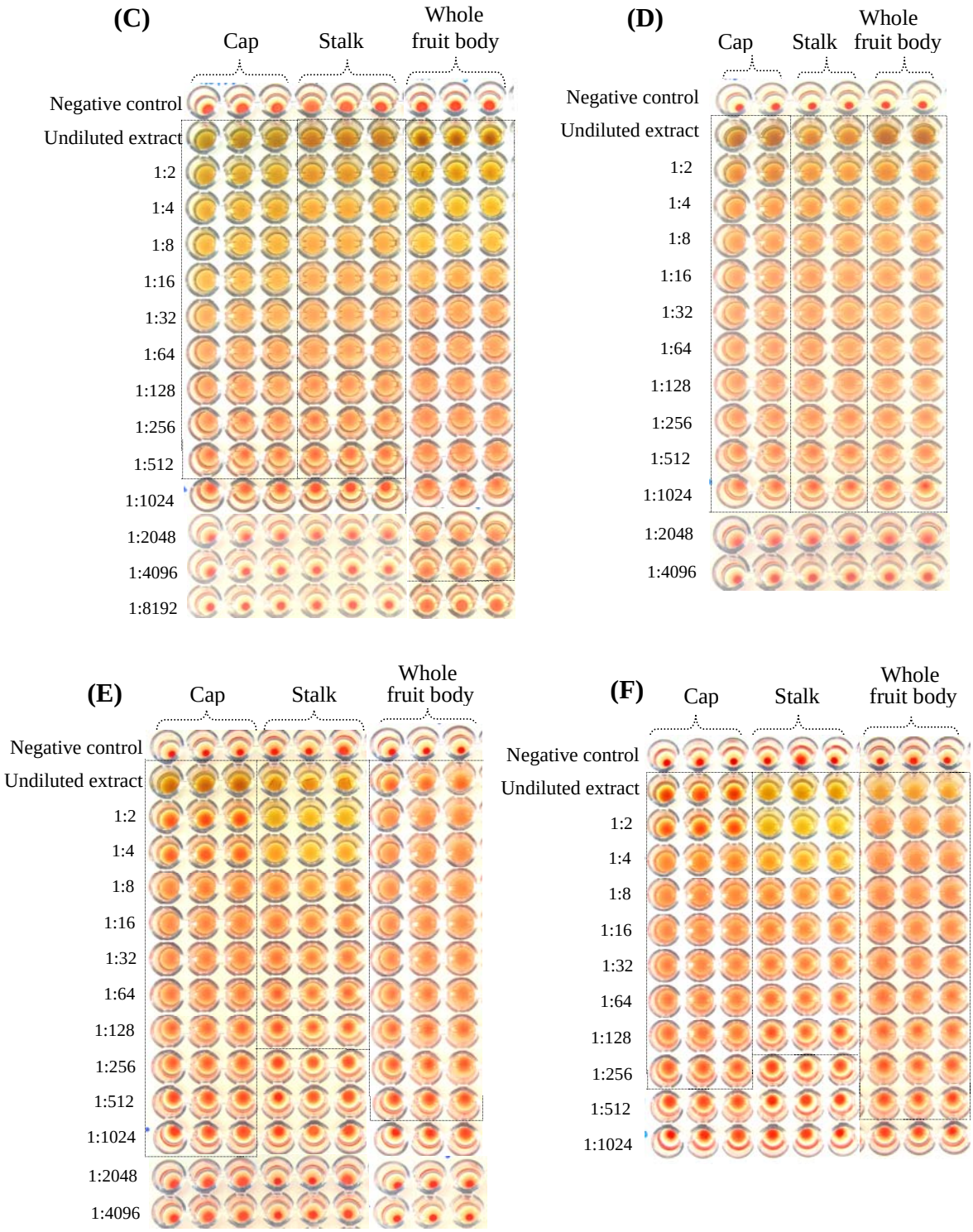

Figure 1C (Continuted) Hemagglutination tests of cap, stalk and whole fruiting body of straw mushroom extracts: MC133 (C), MC134 (D), MC138 (E) and MC139 (F) against rabbit red blood cells.

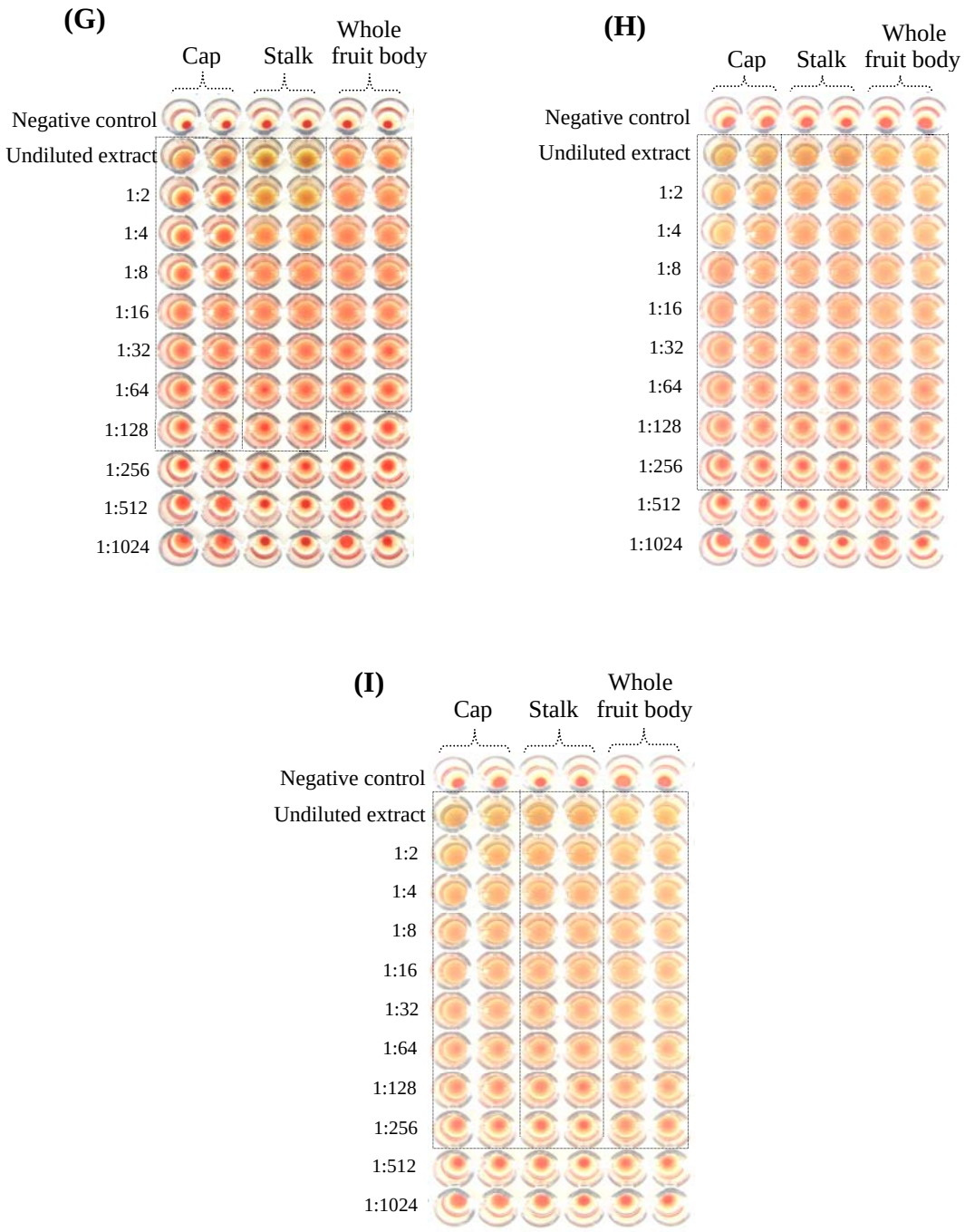

Figure 1C (Continuted) Hemagglutination tests of cap, stalk and whole fruiting body of straw mushroom extracts: MC140 (G), MC144 (H) and MC145 (I) against rabbit red blood cells.

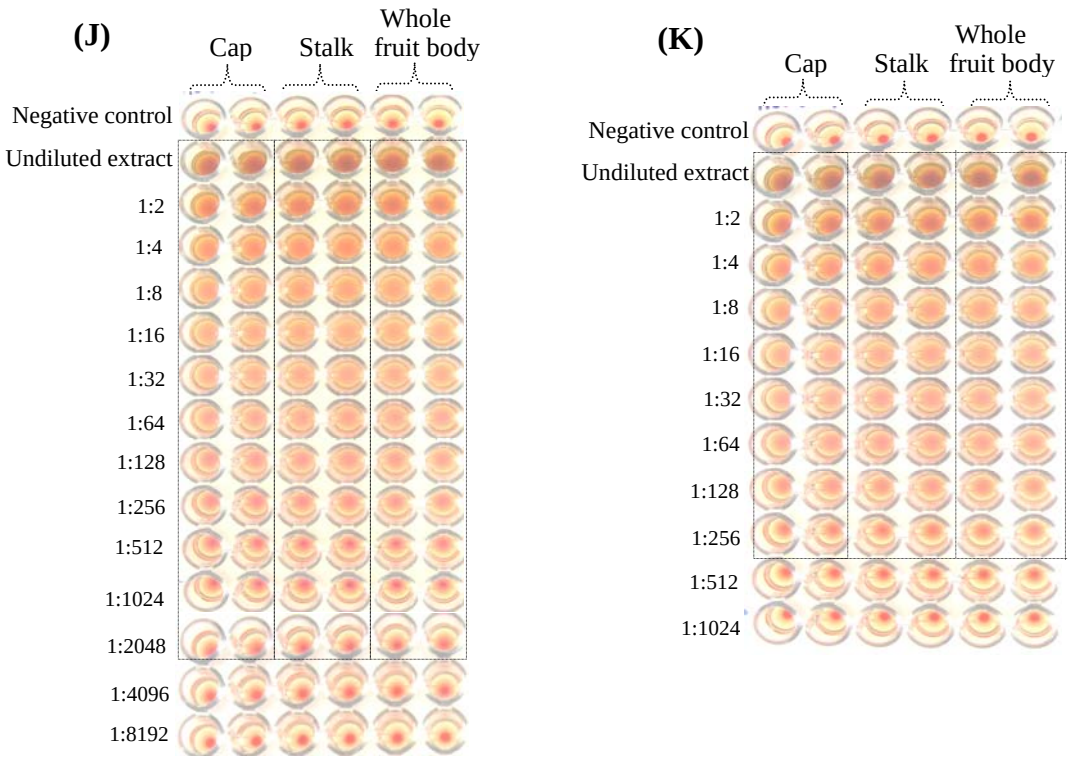

Figure 1C (Continuted) Hemagglutination tests of cap, stalk and whole fruiting body of straw mushroom extracts: MC167 (J) and MC168 (K) against rabbit red blood cells.

APPENDIX D

NUCLEOTIDE SEQUENCE DATA

Nucleotide sequence results of 18S rRNA gene of straw mushroom MC131 were presented in electrophenogram.

Figure 1D Electrophenogram of 18S rRNA gene sequence of straw mushroom specimen MC131 using NS1 primer.

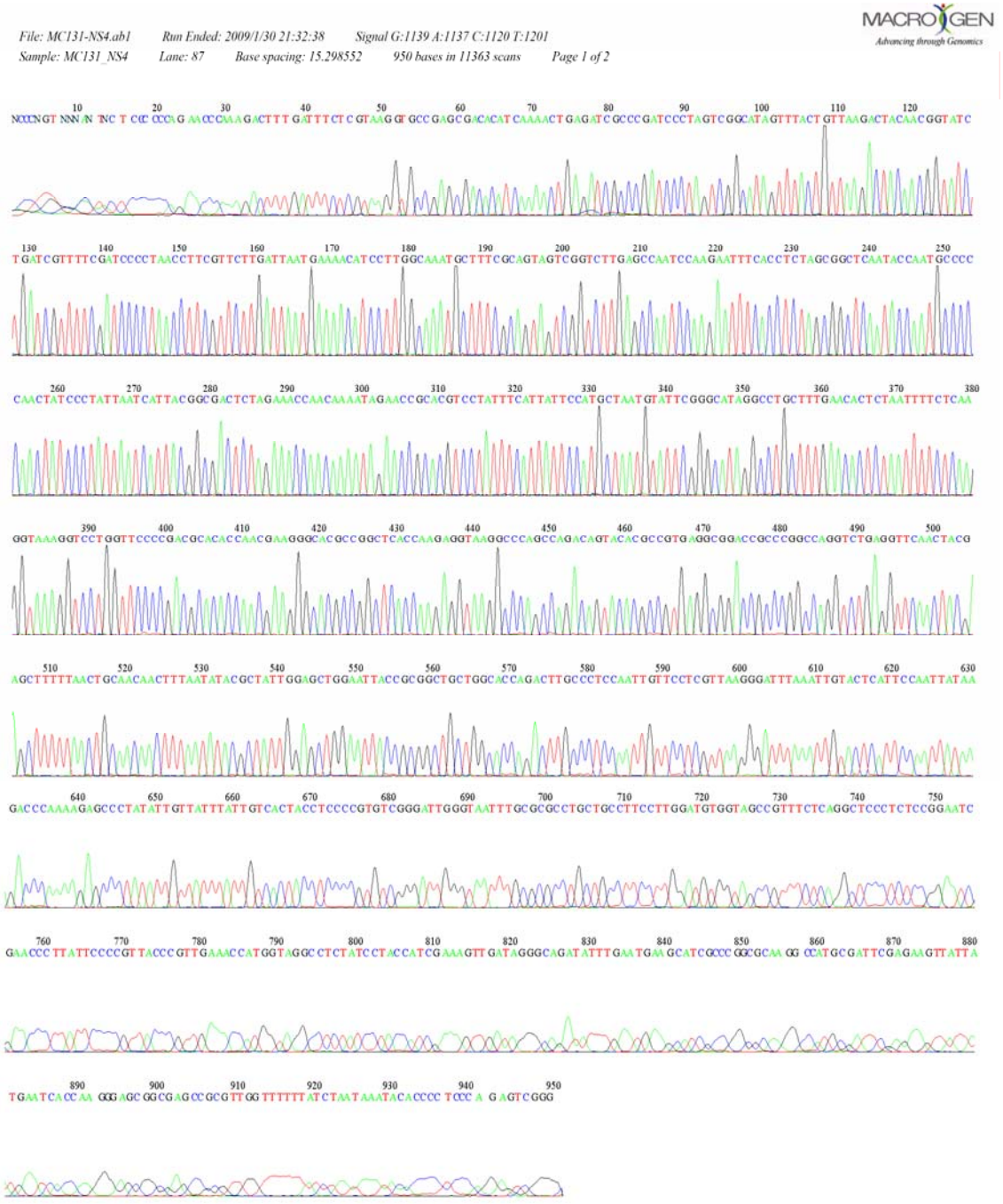

Figure 2D Electrophenogram of 18S rRNA gene sequence of straw mushroom specimen MC131 using NS4 primer.

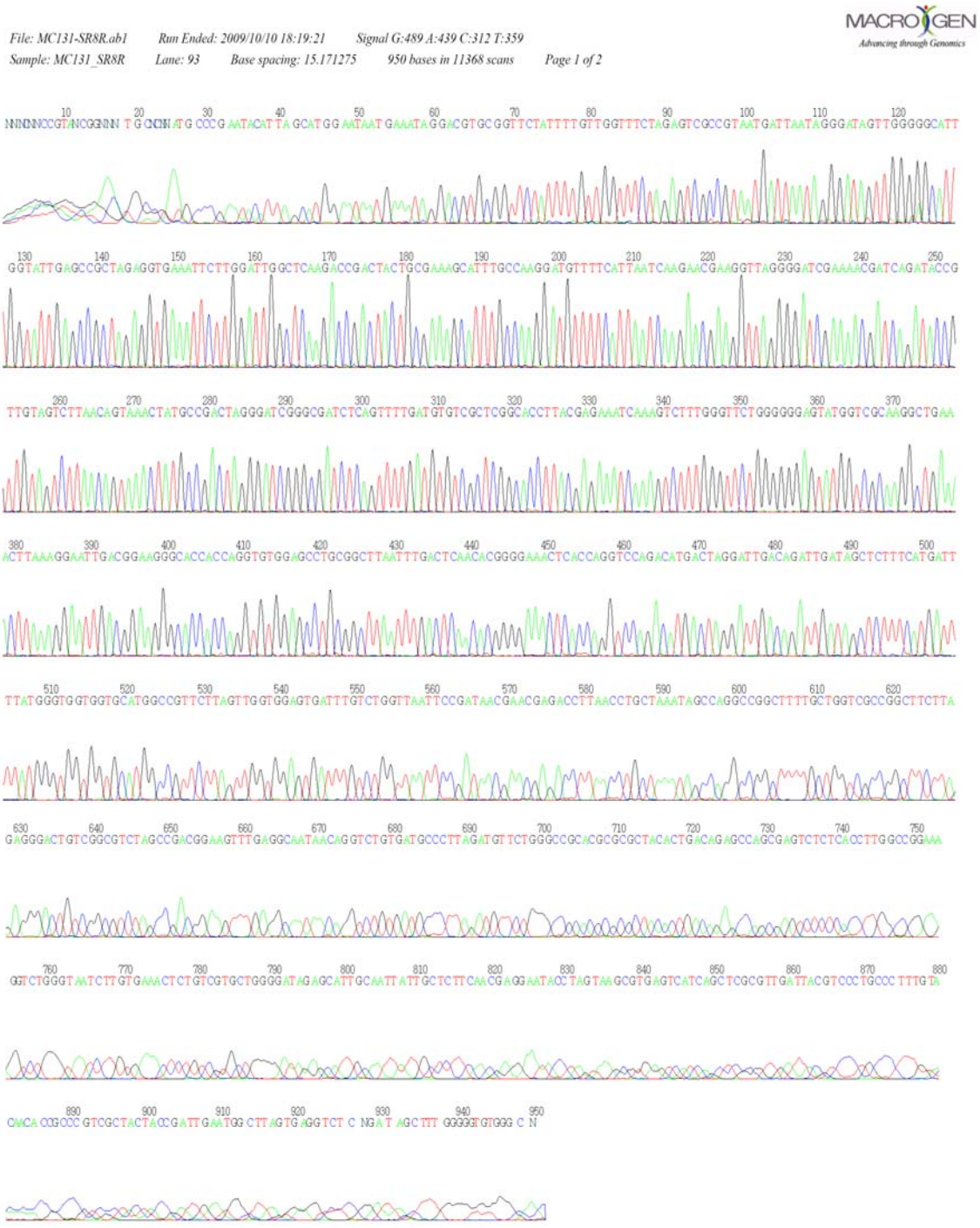

Figure 3D Electrophenogram of 18S rRNA gene sequence of straw mushroom specimen MC131 using SR8R primer.

APPENDIX E

CRYSTALLIZATION AND X-RAY CRYSTALLOGRAPHY EQUIPMENTS

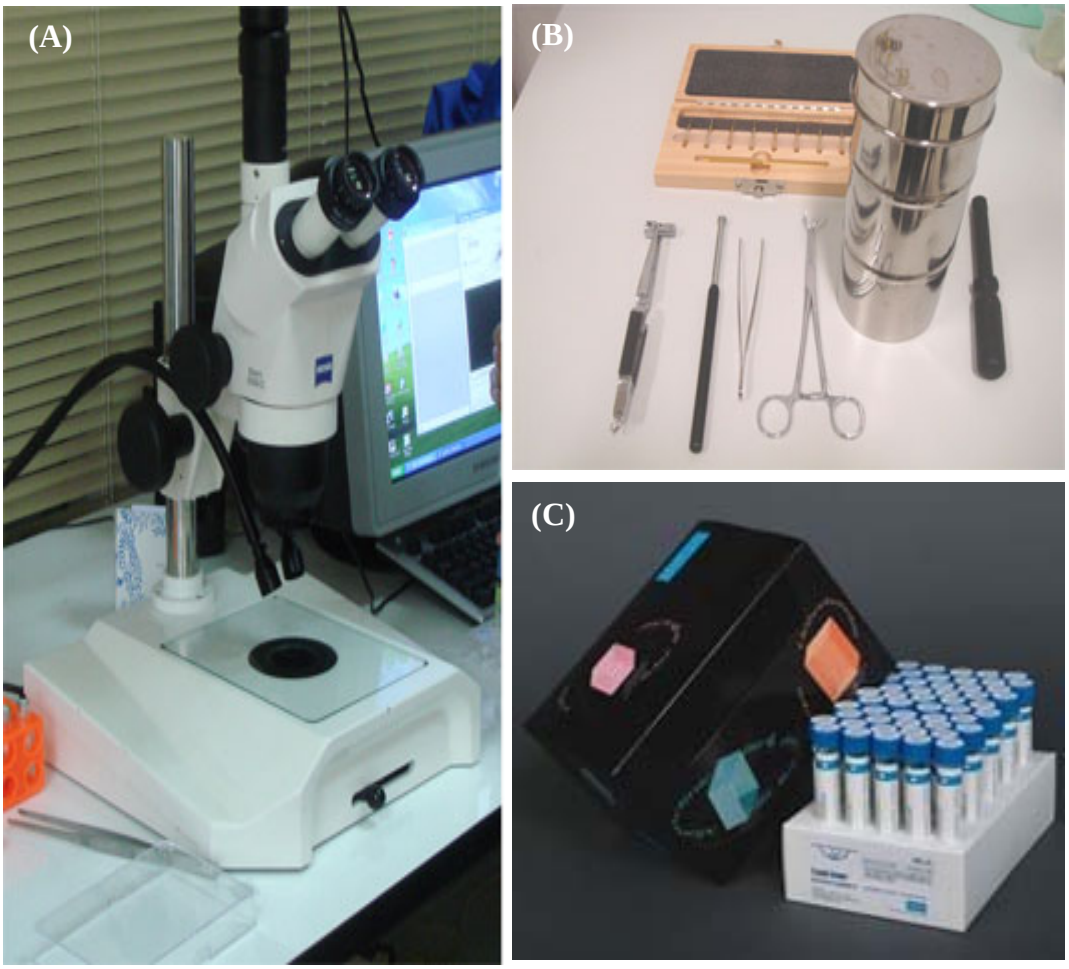

Figure 1E Equipments for crystallization; A, sereomicroscope; B, tools for sample preparation (forceps, crystalcaps, cryoloops, cryotong); C, cystal screening kit reagents from (Hampton Research, U.S.A).

Figure 2E PX diffractometer consisting of rotating anode, CCD detector, Oxford cryostream, and the goniostat on the Macromolecule Crystallography end station at the Synchrotron Light Research Institute (Public Organization) (SLRI), Thailand.

APPENDIX F

LIST OF PRESENTATIONS

Poster Presentation

Mothong, N., Songsiriritthigul, C., Roytrakul, S., and Rodtong S. (2009). **Characterization of a monomeric lectin from the edible mushroom *Volvariella volvacea*.** The 21st Annual Meeting and International, September 24-25, 2009, Queen Sirikit National Convention Center, Bangkok, Thailand.

Mothong, N., Songsiriritthigul, C., and Rodtong, S. (2009). **Variation in lectin accumulation of straw mushroom fruit bodies collected from different locations.** The 2nd SUT Graduate Conference, January 21-22, 2009, Suranaree University of Technology, Nakhon Ratchasima, Thailand.

Mothong, N., Songsiriritthigul, C., and Rodtong, S. (2008). **Purification and characterization of lectin from straw mushroom.** The 3rd Annual Meeting of Thai Mycological Association and Mycology Conference, October 11, 2008, Khon Kaen University, Khon Kaen, Thailand.

Proceeding

Mothong, N., Songsiriritthigul, C., and Rodtong S. (2010). **Crystallization and x-ray diffraction studies of a low molecular weight-monomeric lectin from straw mushroom.** The 5th Annual Symposium of Protein Society of Thailand

“Protein Research: From Basic Approaches to Modern Technologies”; Bangkok, Thailand, June 23-25, 2010, Convention center, Chulabhorn Research Institute Bangkok, Thailand.

The 21st Annual Meeting and International Conference of Thai Society for Biotechnology
TSB 2009: "Biotechnology: A Solution to the Global Economic Crisis?"
24-25 September 2009: Queen Sirikit National Convention Center, Bangkok, Thailand

P-AG12

CHARACTERIZATION OF A MONOMERIC LECTIN FROM THE EDIBLE MUSHROOM *Volvariella volvacea*

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Lectins are proteins or glycoproteins of non-immune origin, which are able to agglutinate cells. *Volvariella volvacea*, commonly known as the straw mushroom belongs to the family Pluteaceae of the Basidiomycetes, which is an edible mushroom of the tropics. It is widely cultivated in Thailand and could be served as a source of lectin. In this study, fruiting bodies of *Volvariella volvacea*, collected from a local market in Nakhon Ratchasima Province, were extracted, purified and characterized. Crude lectin was extracted from fruiting bodies of *Volvariella volvacea* using 10 mM phosphate buffer saline (PBS, pH 7.4), and detected its unique properties by hemagglutination assay using rabbit erythrocytes. Crude lectin extract (1:10 dilution) showed high affinity toward rabbit erythrocytes and displayed inhibitory activity against *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 29213, and *Escherichia coli* ATCC 25922. The *Volvariella volvacea* extract was purified using a procedure that involved ion exchange chromatography on DEAE-sepharose and gel filtration by fast protein liquid chromatography on Superdex 75, and then the purified mushroom lectin was characterized for its physical and chemical properties. Under reducing and non-reducing conditions, SDS-polyacrylamide gel electrophoresis gave a major band of 25 kDa. The *Volvariella volvacea* lectin, which is a glycoprotein, was stable at temperatures up to 60 °C for 30 min and at pH 7 for 18-h. For protein identification, the purified 25 kDa protein band from the SDS-PAGE was excised, in-gel digested and the tryptic peptides were analyzed with liquid chromatography/electrospray ionization mass spectrometry. This process, commonly denoted as MS/MS ion search, showed an unambiguous match of this protein to the Volvatoxin A2. The purification technique, physical, chemical and biological property data of the *Volvariella volvacea* lectin achieved were applied for further investigation and applications.

Key word: Monomeric lectin, Straw mushroom, *Volvariella volvacea*, Characterization

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Variation in lectin accumulation of straw mushroom fruit bodies collected from different locations

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Some certain mushroom species can produce lectins, which are di- or multi-valent carbohydrate-binding proteins or glycoproteins of non-immune origin. The proteins are able to agglutinate cells, and exhibit potential activities such as antitumor, mitogenic, immunoenhancing, and vasorelaxing activities. In this study, the accumulation and properties of lectins in fruit bodies of straw mushroom, Volvariella volvacea, collected from different locations in North-eastern Thailand, were investigated. A total of eleven straw mushroom specimens were collected from 9 provinces. Crude lectins were extracted from cap and stalk of mature fruit body, and from whole fruit body of young specimen using phosphate buffer saline (pH 7.4), then detected their unique properties by hemagglutination assay using rabbit erythrocytes. Crude lectin extracts (1:10 dilution) from cap, stalk, and whole fruit bodies displayed hemagglutination titers in the range of 256 to 4,096, 256 to 2048, and 256 to 4096, respectively. Only extracts of three mushroom specimens showed their inhibition activity against Bacillus subtilis ATCC 6633, Staphylococcus aureus ATCC 29213, and Escherichia coli ATCC 25922. Two extracts having antibacterial activity were successfully purified by anionic exchange chromatography using diethylaminoethyl (DEAE)-Sepharose resin. All purified lectins appeared as a single band with a molecular mass of 25 kDa in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). These results reveal the variation in lectin accumulation of straw mushroom fruit bodies collected from different locations in North-eastern Thailand, and achieved data will be applied for further investigation and applications.

Keywords:

* Corresponding author. *E-mail*: M5010196@g.sut.ac.th

The 3rd Annual Meeting of Thai Mycological Association and
Mycology Conference in Thailand. 11 October 2008, p. 45.

Purification and Characterization of Lectin from Straw Mushroom

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Abstract: From a variety of natural habitats and forests in Thailand, it provided a great biological activities and diversity of edible mushrooms. Lectins, extracted from some mushroom species, exhibit antitumor activity as well as other potential activities such as mitogenic, immunoenhancing, and vasorelaxing activities. Therefore, the tropical mushrooms can serve as a promising alternative source for medicinal lectin investigation. In this study, lectin accumulated in fruit bodies of straw mushroom, *Volvariella volvacea*, collected from Nakhon Ratchasima Province, was extracted, purified and characterized. Hemagglutination assay using rabbit erythrocytes was applied for lectin property detection. The straw mushroom lectin was successfully purified by ion exchange chromatography using DEAE-Sepharose resin. It displayed high specificity against rabbit erythrocytes. The mushroom lectin was stable at temperatures up to 65^{O}C for 30 min, and was not inhibited by D-galactose, D-glucose, fucose, and *N*-acetyl-D-galactosamine at the concentration of 250 mM. In addition, the mushroom extract showed inhibition activity against *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 29213, and *Escherichia coli* ATCC 25922. The purified lectin appeared as a single band with a molecular mass of 25 kDa in SDS–PAGE. The suitable purification technique, physical, chemical and biological property data of the straw mushroom lectin achieved were applied for further investigation and applications.

Key words: Lectin, straw mushroom, *Volvariella volvacea*, purification, characterization

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Proceedings 4

CRYSTALLIZATION AND X-RAY DIFFRACTION STUDIES OF A LOW MOLECULAR WEIGHT-MONOMERIC LECTIN FROM STRAW MUSHROOM

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ABSTRACT

Lectins are di- or multi-valent carbohydrate-binding proteins or glycoproteins of non-immune origin, which are able to agglutinate cells. Some certain mushroom species can produce lectins. The straw mushroom, *Volvariella volvacea*, is edible and is widely cultivated in Thailand. It has been reported that straw mushroom accumulates lectins in its fruit bodies. In this study, lectin accumulated in fruit bodies of straw mushroom cultivated in Nakhon Ratchasima Province was extracted, purified, and crystallized. A low molecular weight-monomeric lectin named MC131 lectin with a molecular weight of 25 kDa was purified from fruit bodies of straw mushroom specimen MC131. The MC131 lectin was crystallized using hanging-drop vapor-diffusion technique. The plate-like crystals of MC131 lectin were successfully grown using the following condition: 5 mg/ml of lectin in the buffer composed of 20% (w/v) polyethylene glycol 20,000 in 0.1 M MES pH 6.5 with incubated at 18 °C for 2 months. Preliminary crystallographic analysis showed that the MC131 lectin crystals belong to the monoclinic P2 system with unit-cell parameters $a = 68.65$, $b = 80.03$, and $c = 105.98$ Å, $\alpha = 90.0°$, $\beta = 106.4°$, and $\gamma = 90.0°$. The crystal could diffract to 3.0 Å resolution, which performed as X-ray diffraction experiments at the Synchrotron Light Research Institute (SLRI), Thailand. This study showed that the straw mushroom could provide lectin crystals for structural biological analysis. Works are underway to improve the resolution, and the crystal quality of lectin.

INTRODUCTION

Lectins are proteins or glycoproteins of non-immune origin, and exhibit a lot of important exploitable biological activities including antitumor, antiproliferative, mitogenic, anti-HIV-1 reverse transcriptase, and immunoenhancing activities [1]. Thailand is one of the potential countries for mushroom lectin investigation. These mushrooms could provide an alternative source of lectins. The straw mushroom, *Volvariella volvacea*, belongs to the family Pluteaceae of the Basidiomycetes, which is an edible mushroom of the tropics. It is widely cultivated in Thailand and could be served as a source of lectins. In this study, lectin accumulated in fruit bodies of straw mushroom cultivated in Nakhon Ratchasima Province, was extracted, purified, and crystallized.

Keywords: Lectin, Crystallization, Straw mushroom, *Volvariella volvacea*, X-ray diffraction

MATERIALS AND METHODS

Purification

The efficiency of purification methods of straw mushroom lectins were precipitated with 90% $(NH_4)_2SO_4$ and chromatographies by ion exchange and gel filtration. Dried fruit bodies of straw mushroom specimen MC131 obtained from Phimai District, Nakhon Ratchasima Province, was extracted with phosphate buffer saline, pH 7.4 containing 1 mM benzamidine, 0.1% 2-mercaptoethanol and 1.5% (w/v) insoluble polyvinylpyrrolidone (PVPP), and purified by 90% $(NH_4)_2SO_4$ precipitation, anion exchange chromatography on a DEAE-sepharose column, and gel filtration by fast protein liquid chromatography on a Superdex 75 column. The purified protein and its molecular weight were determined on 12.5% SDS-PAGE. Protein concentration was estimated by the method of Bradford *et al.* [2].

Crystallization

The purified MC131 lectin at concentration of 25 mg/ml was used for screening crystallization conditions using the microbatch under oil technique with Hampton Research Crystal Screen HR2-110 and HR2-112 (Hampton Research, California, U.S.A.) at 18 °C. Based on the results of crystallization screening, some parameters, particularly protein concentration, ammonium sulphate salt concentration and pH of crystallization solution, must be optimized for crystal growth. The purified lectin concentration of 5 mg/ml and the hanging drop vapor diffusion method were used for further optimization of growing crystals of lectin.

Preliminary X-ray diffraction analysis

Trial exposures of the lectin crystal were performed as X-ray diffraction experiments, which were carried out at 100 K using a Microstar™ rotating-anode X-ray generator at the Synchrotron Light Research Institute (SLRI), Thailand, and a Mar165 charge-coupled device (CCD) detector on the MX end station. The X-ray diffraction data were collected at a wavelength of 1.542 Å.

RESULTS

MC131 lectin was purified from fruit bodies of straw mushroom, *Volvariella volvacea*, obtained from Phimai District, Nakhon Ratchasima Province (Figure 1) by anion exchange chromatography on a DEAE-sepharose column and gel filtration chromatography on a Superdex 75 column. It appeared as a single band with a low molecular weight of 25 kDa in SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing condition (Figure 2A). The molecular weight of native lectin estimated by gel filtration based on the standard markers was equal to the denatured lectin estimated by SDS-PAGE, which a low molecular weight of 25 kDa (Figure 2B). These result indicated that native of MC131 lectin was most likely a monomeric lectin.

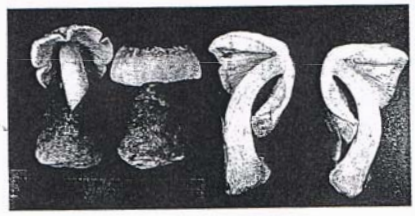

Figure 1 Morphology of fresh fruit bodies of straw mushroom specimen MC131, obtained from Phimai District, Nakhon Ratchasima Province

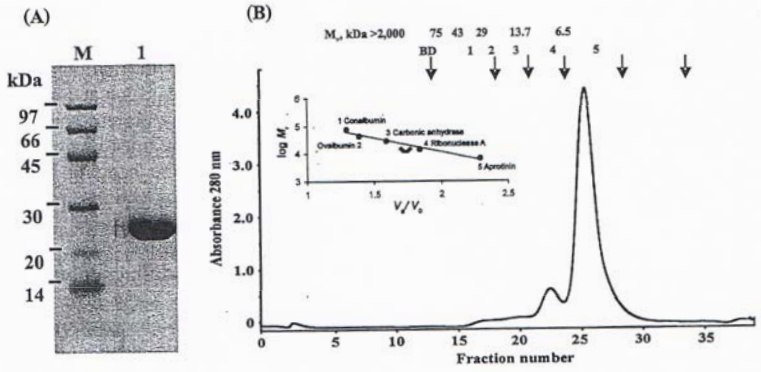

Figure 2 Analysis of purified MC131 lectin by SDS-PAGE and gel filtration chromatography. (A) SDS-PAGE (12.5%) of purified MC131 lectin. Lane M, LMW protein markers (GE Healthcare); Lane 1, purified MC131 lectin. (B) elution profile of MC131 lectin using gel filtration chromatography on a Superdex 75 column 10/300 GL by FPLC on an AKTA Purifier, which was eluted with 10 mM Tris-HCl pH 7.3 containing 100 mM NaCl. The flow rate was 0.5 ml/min. Elution positions of gel filtration calibration kit LMW (GE Healthcare) were indicated by arrows. BD, blue dextran (>2,000 kDa); 1, conalbumin (75 kDa); 2, ovalbumin (43 kDa); 3, carbonic anhydrase (29 kDa); 4, ribonuclease A (13.7 kDa); 5, aprotinin (6.5 kDa). The inset displayed the obtained calibration curve determining the M_r of MC131 lectin

Six out of 98 conditions provided needle-like and plate-like crystals within 1 and 3 days of incubation at 18 °C. These conditions were then further optimized for the conditions for crystal growth using hanging drop vapor diffusion method. The MC131 lectin crystal growth was successfully obtained with conditions: 5 mg/ml of lectin in the buffer composed of 20% (w/v) polyethylene glycol 20,000 in 0.1 M MES pH 6.5 with incubated at 18 °C for 2 months. This condition gave plate-like crystals (Figure 3A). The preliminary X-ray diffraction clearly showed that the crystal of MC131 lectin was protein crystal and could diffract to 3.0 Å resolution (Figure 3B). Analysis of the collected data indicated that MC131 lectin crystals belong to a

monoclinic P2 system with unit-cell parameters a = 68.65, b = 80.03, and c = 105.98 Å, α = 90.0°, β = 106.4°, and γ = 90.0°.

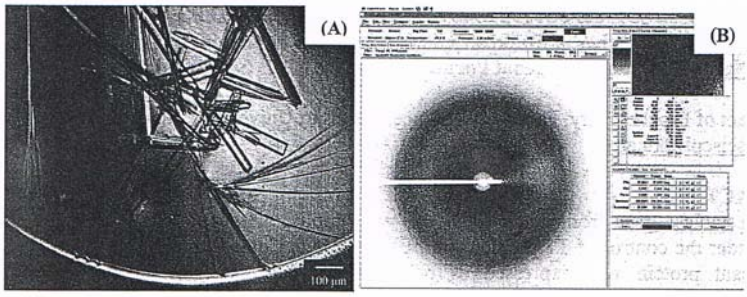

Figure 3 Crystal appearance and X-ray diffraction pattern of MC131 lectin. (A) the plate-like crystals of MC131 lectin were grown in condition: 5 mg/ml of lectin in the buffer composed of 20% (w/v) polyethylene glycol 20,000 in 0.1 M MES pH 6.5 with incubated at 18 °C for 2 months. (B) X-ray diffraction pattern from a crystal of MC131 lectin

CONCLUSIONS AND DISCUSSION

A low molecular weight-monomeric lectin with a molecular mass of 25 kDa was successfully purified from fruit bodies of straw mushroom specimen MC131 obtained from Phimai District, Nakhon Ratchasima Province, by using anion exchange chromatography on a DEAE-sepharose column and gel filtration chromatography on a Superdex 75 column. The straw mushroom could provide lectin crystals for structural biological analysis. From the X-ray diffraction pattern point of view, higher resolution, crystal size, and better quality must be obtained. More protein crystallization trials need to be performed and the preliminary conditions should be finely optimized. X-ray diffraction experiments must be carried out with care especially during the mounting process.

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