

國立交通大學生物科技系
博士論文

利用分群分析香菇菌種類源與多醣結構的特色及利用多樣統計分
析香菇多醣體結構與巨噬細胞活性關係之研究

**Elucidation of Strains Grouping and Polysaccharide
Structural Features by Cluster Analysis and the
Relationships of Polysaccharide Structure-Macrophage
Stimulatory by Various Statistical Analyses on Ten
Regionally Different Strains of *Lentinula edodes***

研究生：羅千婷

Student: Tiffany Chien Ting Lo

指導教授：張正 教授

Advisor: Prof. C. Allen Chang

中華民國九十六年五月

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研究生：羅千婷

Student : Tiffany Chien-Ting Lo

指導教授：張正

Advisor : Cheng Allen Chang



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中文摘要

本研究利用深層培養十株不同的香菇菌種，將菌種培養至靜止期後萃取其發酵濾液中的多醣體進行單醣組成、分子量分布、結構型態、免疫調節活性及抗腫瘤活性的測定。測定出十株菌種的多醣體皆為 α -(1 \rightarrow 4)-glucan 鍵結 β -(1 \rightarrow 6)-glucan 複雜巨分子，分子量分布在 1×10^2 至 3×10^3 kDa，並且各由不同比例的七種單醣所構成，包括了：葡萄糖、甘露糖、木糖、半乳糖、黑藻糖、鼠李糖及阿拉伯糖。由於固態的多醣體具有不易回溶水的特性，因此我們開發以超臨界流體系統之高壓水方式，在 10.1MPa、28°C、70 分鐘的條件下提升多醣體的回溶率可達 90%，而且此萃取方法依然能保 α -(1 \rightarrow 4)-glucan 鍵結 β -(1 \rightarrow 6)-glucan 的結構及其生物的活性。我們也發現 α -(1 \rightarrow 4)-glucan 鍵結 β -(1 \rightarrow 6)-glucan 多醣體亦能引發 RAW264.7 巨噬細胞的細胞與細胞間之訊息傳遞。

另外，以多變量迴歸分析十種多醣體中單醣組成與 RAW264.7 巨噬細胞活性的關聯及建立結構-活性預測模式；以主成分分析法和因素分析法探就出阿拉伯糖、甘露糖、半乳糖及黑藻糖為多醣結構中的組成特色。最後再利用擴增片段長度多形性分析菌種的類源及利用統計的群集分析醣鍵結的構型關係，進一步比較基因分群與結構分群的相同或相異之處。多醣體的結構與其引發的生物活性相當複雜，我們的研究結果能助於開啟醣類資訊學的新興研究的方法，也能有助於簡化醣類領域的複雜問題。

Abstract

In this research, we have made major efforts to isolate polysaccharides from the submerged cultures of ten different strains of *Lentinula edodes* and have determined their monosaccharide compositions, molecular weights, structural linkage types, immuno-modulating and anti-cancer activities. The isolates had a molecular weight distribution between 1×10^2 and 3×10^3 kDa. The monosaccharide composition analysis revealed that these polysaccharides were heterogeneous and contained glucose, mannose, xylose, galactose, fucose, rhamnose and arabinose in different ratios. A major form of polysaccharide linkage with a backbone of α -(1→4)-glucan and side chains of β -(1→6)-glucan has been identified. A method using the pressurized water extraction (PWE) technique (10.1 MPa, 28 °C, 70 min) to improve the extraction efficiency and to retain the general structures and bioactivities of the polysaccharides has also been developed. These polysaccharides were also found to stimulate macrophage cell-cell interactions.

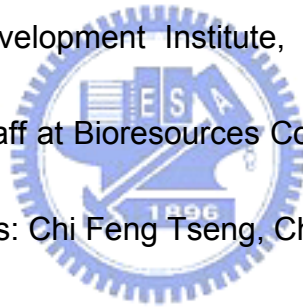
The use of multiple linear regression analysis allows the deduction of the correlation between the monosaccharide composition ratios and *in vitro* macrophage (RAW264.7) stimulatory activities of polysaccharides obtained from different mushroom strains. Arabinose, xylose, mannose, fucose and galactose were identified as the monosaccharides that could be related to macrophage stimulatory activities. In addition, principal component analysis and factor analysis methods were used to find

polysaccharide structural characteristics of these *L. edodes*. Furthermore, investigations by amplified fragment length polymorphism analysis (AFLP) and by statistical cluster analyses of GC-MS linkage data were compared to understand the relationships of genotypes and structural features of these species. The structures and biological activities of these polysaccharides were quite complex and remained to be delineated in the future at the molecular level. The novel feature of using statistical methods to treat limited but complex experimental data in this work represents a new approach in the bioinformatics area and allows the simplification of research problems in the glycobiology field.



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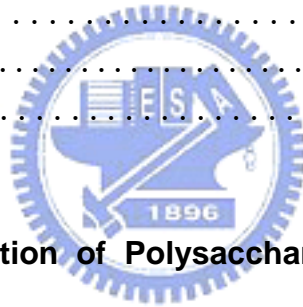
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I . Introduction

Lentinula edodes

Mushrooms such as *rieshi* (*Ganoderma lucidum*), *shiitake* (*Lentinula edodes*) and others have been cultivated and used for many years (1). More than 140,000 different kinds of species, i.e. roughly 10% of available species on earth have been identified (2). They have been valued as flavorful and nutritious foods as well as precious medicines, e.g. as immune-modulators, in the Far East. Among them, the shiitake mushroom, *Lentinula edodes* (*L. edodes*) is the most important cultivated mushroom and is easily available from Korea, Russia, Taiwan, China and Japan (3, 4). Of all the mushroom immune-modulators investigated, bioactive polymers from *L. edodes* have been studied the most (5).



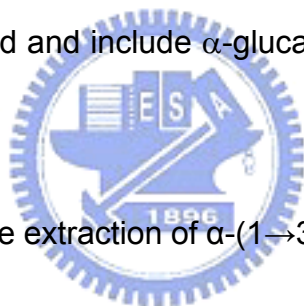
More than 100 cultivated shiitake strains have been developed. Traditional methods to differentiate various strains typing in shiitake cultivars were usually carried out based on morphological, physiological, and somatic compatibility characteristics (6). Recently, additional molecular techniques were developed. For example, Vos *et al.* (7) developed AFLP (amplified fragment length polymorphism) fingerprinting analysis based on selective polymerase chain reaction (PCR) amplification of genomic DNA restriction fragments and demonstrated that AFLP analysis could reproducibly help identifying a large number of polymorphic loci to differentiate various

strains (7). Applications of AFLP analysis (fingerprinting) for population studies have become quite popular and important, and have been reported frequently (8, 9). The technique could successfully differentiate not only the diversity in flowering plants populations but also a wide variety of organisms (10, 11, 12). For example, AFLP marker techniques have been successfully used to derive the genetic linkage map of *L. edodes* for frozen material and heat dried fruiting bodies (13, 14).

Polysaccharides attract immunomodulatory activities

The basic composition of mushrooms includes vitamins, minerals, phenolic compounds, total carbohydrates, dietary fiber, nitrogen, and protein that vary considerably in different strains (15, 16). According to Crisan *et al.* and Bano *et al.*, the carbohydrate contents of *L. edodes* varied from 67.5% to 78% on a dry weight basis (17, 18). The carbohydrates in mushrooms include polysaccharides such as glucans, mono- and di-saccharides, sugar alcohols, glycogen, and chitin (19). Of all, β -glucan has been identified as the major structural component among many polysaccharides with anti-cancer activities (20). For example, Lentinan (extracted from cell wall of fruiting body), KS-2 (a α -mannan peptide extracted from culture mycelia) and LEM (a β -glucan-protein complex extracted from solid medium) of *L. edodes* are demonstrated to be immuno-potentiators and exhibit anti-cancer activities (21-24). Mueller *et al.*, (25) have reported that binding of glucans to macrophage and

neutrophil cell lines resulted in stimulation of the expression of nuclear factor- κ B (NF κ B). The structural elucidations for Lentinan show the presence of only glucose molecules with mostly β -(1 \rightarrow 3)-glucan linkages in the regularly branched backbone and β -(1 \rightarrow 6)-glucan in side chains (26, 27). The major active constituent of the KS-2 mycelium extract is reported to be a hetero-glycan protein conjugate containing ca. 24.6% protein and 44% monosaccharide in addition to nucleic acid derivatives and vitamins (28). Simultaneously, many other active polysaccharides, protein-polysaccharide conjugates and water-soluble lignin's were isolated (29). However, the reported structures are varied and include α -glucans as well as many hetero-glucans (30-32).



Shida *et al.* reported the extraction of α -(1 \rightarrow 3)-D-glucan from fruiting bodies of *L. edodes* (33, 34). However, not much attention was paid to the properties of this form of glucan. Rather, modified polysaccharides such as those by sulfation, sulfonylation or carboxymethylation that showed high anti-cancer activities gained rapid attention and wide scale applications in recent years (8, 35-37). Specifically, α -glucan and modified α -glucan (e.g. as sulfate) of *L. edodes* were demonstrated to show anti-cancer activities against Sarcoma 180 (*in vivo*) and against six tumor cell lines (*in vitro*, 38). In some cases, unmodified α -glucan failed to inhibit *in vitro* tumor growth (38).

The studies on the structure-activity relationship and diversity of glucans mediated immuno-pharmacological activities have since gained momentum (4, 23, 39, 40). The pharmacological activities of mushroom polysaccharides against tumors have been thoroughly reviewed (41). The preliminary anti-cancer mechanisms of whole mushrooms and isolated polysaccharides are proposed to involve the intervention of macrophages and 'T' cells (42). TNF- α (tumor necrosis factor, a pro-inflammatory cytokine) is then released from macrophages or activated 'T' cells in response to microbes or other agents (43). The detailed mechanisms by which these polysaccharides compounds exert their anti-cancer effects remain largely unexplored despite the established vital role of glucans. We have selected ten regional different *L. edodes* and determined the monosaccharide composition, molecular mass distribution, structural linkages and macrophage-stimulating and of their polysaccharides extracts, hoping to add new and more insights to this important research area.



Extraction of polysaccharides

The isolation of solid polysaccharides from various sources remains as a major problem because they are heterogeneous with low water solubility and the water extraction yield was normally quite low. In our experience, the boiling water extraction (BWE) of the polysaccharides was usually lower than 30% of the obtained crude

products (4, 44-46). Because the submerged cultivating process of *L. edodes* is rather time-consuming and normally takes several weeks, it is always necessary to optimize the polysaccharides extraction yield for further research and application purposes.

Extractions of polysaccharide from mushrooms with boiling water, sodium hydroxide, ammonium oxalate and pulsed electric field were also reported for improving efficiency (44, 47). However, the structures and bioactivities of a number of polysaccharides extracted under these conditions were not compared before and after the extractions. On the other hand, extractions with supercritical fluids at high pressures have been demonstrated to be quite useful for many food and biological samples (48-53). The few reports were published on the use of pressurized water extraction (PWE) for polysaccharides from *L. edodes*. We reported that the PWE method could improve the extraction efficiency and could retain macrophage stimulating activity of the polysaccharides.

Glyco-informatics

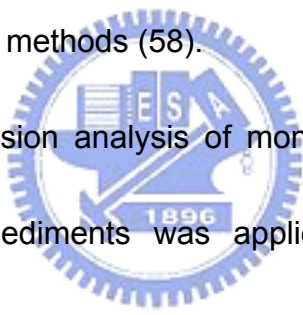
Different strains of higher *Basidiomycota* mushrooms are known to produce biologically active polysaccharides with different properties and chemical structures. The structures include mainly glucan and glycan normally synthesized biologically to form tree structures with various types of branched linkages (54). Glucan and glycan play key roles in cell communication, protein interaction and immunity. Various

monosaccharides form the nodes of the tree structures and with glycosidic bonds leading to branched linkages. For each monosaccharide there are six possible hydroxyl groups for linkage formation and two possible anomality 'α' or 'β' could result (55).

Polysaccharides isolated from the different strains of *Poria cocos* mycelia showed different *in vivo* and *in vitro* anti-tumor activities, depending on their monosaccharide composition, molecular mass, and chain conformation (56). In the research, we reported the monosaccharide composition, molecular mass, structural linkage, immuno-modulating and anti-tumor activities for polysaccharides extracted from different phylogenetic groups of ten regional *L. edodes* (57). The immuno-modulating properties and anti-tumor activities of these *L. edodes* extracts were tested and the results showed that the ten isolated *L. edodes* could be classified into three distinct groups using amplified fragment length polymorphism assay. All polysaccharides had similar molecular mass distribution between 1×10^4 and 3×10^6 . The monosaccharide composition analysis revealed the presence of heterogeneous materials containing glucose, mannose, xylose, galactose, fucose, rhamnose and arabinose in different ratios. Most of the extracts exhibited significant enhancement in macrophage stimulatory activities (MSA). However, although several studies were reported concerning the immunological activity and structure of polysaccharides (mostly β-glucan) in regionally different *L. edodes*, the determining factors for the

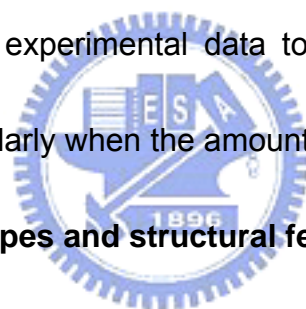
structure-function relationships remained unsolved.

Many methods in applied mathematics and statistics are often used to summarize and/or simplify complex data and help deducing relationships among variables. Regression analyses have been broadly employed to predict sources or structure-function relationship in biological sciences. Examples include the utilize of regression analyses to predict the distribution of tree and shrub species, terrestrial animal species (58), drug discovery (59), and culture conditions of *L. edodes* (60). However, for different areas and/or at different resolutions, it may be difficult to compare the results of these methods (58).



A simple linear regression analysis of monosaccharide (arabinose, mannose and xylose) contents in sediments was applied to determine the sources of carbohydrates (61). In addition, principal component analysis and factor analysis could be constructive in describing and classifying different functional or structural groups, for the measure of glycan structures taking into account of species, for the exploration of molecular structural characteristics, for the evaluation of the polysaccharide composition in lignified woody plant cell wall, for the evaluation of molecular lipophilicity, for the understanding of organic reaction mechanisms, for quantitative structure-retention studies in chromatography, for quantitative structure-biodegradatoion relationship, and for classification of drug-free subject and drug

abuses groups employ the metal contents in hair samples (54, 59, 62). For example, Hori *et al.* used a combination of FT-IR microscopic techniques and principal component analysis method to investigate the chemical variations between softwood species as well as types of wood cell walls (63). Hizukuri *et al.*, applied the principal component analysis to the composition data and found that the compositions of mannose, xylose and arabinose were important characteristics that distinguished the difference between yeast, sycamore and wheat (55). To the best of our knowledge, there have been few reports applying more than one statistical methods for a comprehensive analysis of experimental data to deduce polysaccharide structure-function relationship, particularly when the amount of data are limited (59).



The relationship of genotypes and structural features of these species

In particular, the glycosyl linkages, formed by various monosaccharides at the nodes of the polysaccharide tree formations, would reveal essential structural information and have been mostly elucidated by gas chromatographic and mass spectrometric (GC-MS) techniques. However, the analyses of these linkage data are sophisticated, usually incomplete and difficult, at least because of the following reasons: (1) most polysaccharides are heterogeneous with high molecular weights, (2) there are many combinations of linkage types between any two monosaccharides, and (3) there is a lack of a complete set of standard materials of all linkage types that

could be used as reference standards.

Recently, mathematical and statistical methods have been applied to help understand the monomer composition of polysaccharide characteristics of woody plants and to establish polysaccharide classifications of Vochysiaceae (63, 64).

Multiple linear regression analysis, principal component analysis and factor analysis methods have also been used to find the polysaccharide structural characteristics and their relationships with macrophage stimulatory activity of regionally different strains of *L. edodes* (65). Among these statistical methods, cluster analysis could be employed

to measure strain similarities if some experimental variables (i.e. glycosyl linkages) are potentially highly correlated (64). Cluster analysis is a generic name for multivariate analysis techniques to create groups of objects based on their degree of association (66). Clustering is useful in several exploratory pattern-analysis, grouping, decision-making and machine-learning situations, including data mining, document retrieval, image segmentation, and pattern classification (67).

In this research, we applied cluster analysis to classify GC-MS linkage data. We wish to report the equivalent or difference between the genotypes grouping and glycosyl linkages classification from ten regionally different *L. edodes*.

II . Materials and methods

Strains, media, and growth conditions.

The ten strains of *L. edodes* are: No.135 (L24) and No.939 (L25) from China, Tainung No. 1 “white cap” (L1) and “red cap” (L4) from Taiwan, Japanese 271 (L11, L15), Jongxing 5 (L6), Jongxing 8 (L10), Hey-King-Gang (L21) and Jong-Wen 600 (L23) from Japan.

The culture growth conditions were optimized by first incubating the mushroom (L11) for 3, 5, 7, 12, 14, 17, 19, 21, 24, 26, 28 and 31 days at 26°C. The culture broth of *L. edodes* was obtained from medium (pH 4.5) containing 2% oat, 0.5% yeast extract, 0.1% KH₂PO₄, 0.05% MgSO₄, 0.15% CaCO₃, at 26°C and reciprocating shaking (150 rpm/min) and fermented for 14 days. The cultivation developed in the flasks was transferred to static incubation. The liquid was filtered (*Membrane filter pore size >0.45 μm*), to collect the culture broth filtrate (CBF) and then passed through the autoclave. The mycelia pellets were collected and washed with distilled water. The mycelia pellet dry weight was determined by drying in an oven for 7 days at 60°C.

Polysaccharides isolation.

The CBF was added to three volumes of 95% ethanol and stored at 4°C overnight. The precipitate was collected by centrifugation, followed by washing with

75% ethanol, and then freeze-dried to obtain a culture precipitate. The crude polysaccharide is treated with boiling water to prepare a BWE that was filtered (Milli-pore, 0.22 μm) and collected (>molecular mass 10,000 Da fractions) of for immunoassay and freeze-dried for further analyses.

Amplified fragment length polymorphism analysis (AFLP).

Mycelia were harvested onto filter paper, rinsed with distilled water, and freeze-dried. Genomic DNA for each progeny was extracted by use of the DNeasyTH Plant Mini Kit (*Qiagen, Hilden*) and DNAs were eluted twice from the DNA binding column with 100 μl AE (50 mM NaAcetate pH 5.2, 10 mM EDTA) solution. AFLP analysis was carried out adopting modification of the procedure described by Vos et al., (7), Terashima et al., (13) the instruction manual of the AFLP core Reagent Kit (*Invitrogen*), and the AFLP Microbial Fingerprinting Kit (*Applied Biosystems*). Electrophoresis and detection of amplified fragments were performed using the 373 DNA Genetic Analyzer (*Perkin Elmer*). Each gel track ranged from the size-marker 50 bp band to the 500 bp band. Patterns were normalized with reference to molecular mass of the internal ROX-labeled size standard that was added to each sample. After normalization, the levels of genetic similarity between the AFLP patterns were calculated with Pearson correlation. For cluster analysis of AFLP banding patterns,

the un-weighted pair group method applying arithmetic averages (UPGMA) was used (68). Internal standards of *L. edodes* strains employed are BCRC 36024 (CBS 454.69, IFO 8340) and 36482 (MUCL 28773). Phylogenetic grouping was performed using Bio Numerics software.

Molecular mass determination.

The molecular mass fractions of CBF extract was determined utilizing a gel-chromatographic technique with H₂O (0.3 ml/min) as a mobile phase (Waters Hsp-Gel AQ5.0, Waters 1515 isocratic pump, 717 plus injector and 2410 RI detector). The calibration curve was constructed with standard dextrans (Polymer Standards Service) within the range of molecular mass from 180 to 2,100,000 Da. Four distinct fractions were identified and labeled as A, B, C, and D, accordingly. L15 BWE or PWE was injected into the gel-permeation chromatograph column (Mix D column, PL Aqua-gel-OH), mobile phase being H₂O, and collected five different fractions- A, B, C and D (and E), applying the same fraction collector as described for CBF extraction.

Monosaccharide composition.

Monosaccharide analysis was performed usage the method of Blakeney and Hoebler (69, 70). The Polysaccharides from CBF (2 mg) were completely hydrolyzed


in a sealed tube with 0.3 ml of 2 M trifluoroacetic acid (TFA) at 120°C for 90 min to convert the monosaccharides into alditol acetates. The amounts of neutral monosaccharides in the polysaccharides were analyzed as alditol acetates by gas chromatography (GC, Varian 3800). Myo-inositol (1 mg) was added as an internal standard. The amounts of neutral monosaccharides (polysaccharides contents) were determined using the phenol-sulfuric acid method (14, 71, 72).

Structure analysis.

In order to determine the functional group of the most immunological active strain, crude polysaccharides or BWE were further characterized using FTIR (all strains) and NMR (L15) spectroscopic techniques. The glycosyl linkage composition was analyzed using gas chromatography (all strains). The FTIR spectra were obtained from 1 mg of crude polysaccharides in 100 mg of KBr. Twelve scans were accumulated with a resolution of 2 cm⁻¹ in the range 500-1800 cm⁻¹ (Jasco 8900) (73-75). Glycosyl linkage composition analysis was carried out by taking 1 mg of crude polysaccharides that were methylated using the modified NaOH-DMSO (dimethyl sulfoxide) method (13, 73, 75, 76). The per-methylated products were hydrolyzed, reduced, acetylated and then analyzed by GC-MS (HP 6890/MSD 5973) at 25°C.

The ^1H , ^{13}C NMR and DEPT (Distortionless Enhancement by Polarization Transfer) experiments were recorded at 600 and 150 MHz respectively, on a Bruker DMX-600 spectrometer (71, 73, 77). The exchangeable proton in BWE residue was exchanged with deuterium by adding sufficient D_2O (99.96% atom^2H , Aldrich). The ^1H and ^{13}C NMR spectra were recorded at 25°C . Methanol was used as an internal standard (δ 49 ppm) for the ^{13}C spectrum. The ^1H NMR spectrum was recorded by adjusting the HOD signal at δ 4.68 ppm. 2D-NMR experiments (COSY and HSQC) were performed by using the standard XWIN-NMR 3.5 software.

Macrophage stimulating activity assay.

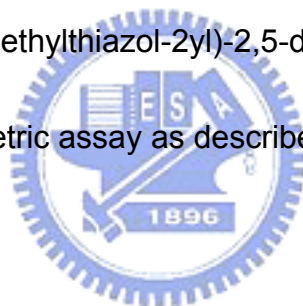


The O_2^- production of macrophage cell line (Mouse BALB/C macrophage, RAW 264.7) was measured by a modified nitroblue tetrazolium (NBT, Sigma) reduction assay. The RAW 264.7 was cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% fetal bovine serum (FBS, Hyclone). Macrophage cell lines were placed into wells of a 96-well microtiter plate (2.5×10^5 per well) and were treated with 20 μl of polysaccharide extracts for 48 h at 37°C . After removal of the supernatant, the macrophage cell line monolayer was covered with 100 μl of 2 mg ml^{-1} of NBT. The plates with stimulated cells were incubated for 4 h at 37°C . The reduced formazan within macrophage cell line was solubilized in DMSO (Merck). Optical density was

measured using an ELISA reader at 570 nm (78, 79). *L. edodes* culture medium was used for control experiments and PBS (phosphate-buffer saline) as blank solution for optical density experiments. The % NBT reduced is estimated utilizing the equation:
$$\frac{[(\text{Sample Average}) - (\text{Blank Average})]}{[(\text{Control Average}) - (\text{Blank Average})]} \times 100.$$

Preparation of 'T' cell.

T-cell line (J45.01, Human acute 'T' cell) was placed into wells of a 96-well microtiter plate (9 x 10³ per well) and was treated with 20 µl of CBF for 72 h. The determination of 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) was performed by a colorimetric assay as described in Tada *et al.*, 1986 (80).



TNF-α release activity.

The ability of CBF of *L. edodes* strains L15 and L23 to stimulate production of tumor necrosis factor-α (TNF-α) by mouse BALB/C macrophage cell line (RAW 264.7) was investigated. The RAW 264.7 cells were cultured in DMEM containing 10% FBS. Macrophage cell lines were placed into wells of a 24-well microtiter plate (2.5 x 10⁵ per well) and stimulated with or without CBF (negative control) or lipo-polysaccharide (Sigma, 10 µg/ml) for 5 to 24 h in a humidified 5% CO₂, 37°C incubator. After incubation, culture supernatant was collected by centrifugation and measured using a

TNF- α Kit (Entogen) in ELISA system (MRX, Dynex). The control experiments were conducted using PBS (Dulbeccos, Ca²⁺/Mg²⁺ free, D-PBS, GibcoBRL 21600-010).

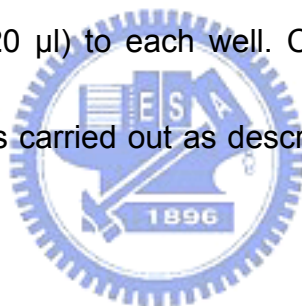
The positive control assay was conducted using lipo-polysaccharide.

Direct anti-cancer activity assay.

Normal cell line (MRC-5) of human embryonal lung cell-density of 3×10^3 , human cervical carcinoma cancer cell line (Hela), cell density of 1.5×10^3 , human gastric cancer cell line (AGS), cell density of 3×10^3 per well, human hepatocellular carcinoma cell line (Hep G2), cell density of 3×10^3 per well, human breast cancer cell line (MCF-7), cell density of 3×10^3 per well were placed into wells of a 96-well microtiter plate and were treated with 20 μ l of CBF (all strains) or HWE (L15 only). The plates with stimulated cells were incubated for 72 h at 37°C. MTT solution was prepared in PBS at 5 mg/ml concentration. This MTT solution (20 μ l) was added to each well. It was further incubated for 4 h. Thereafter, the supernatant was aspirated carefully from each well, and the formazan crystals were dissolved in DMSO (Merck). Optical density was measured using ELISA reader at 540 nm. Then the % viability was calculated in comparison to control, taking control (culture *L. edodes* medium) as 100% viable.

Indirect anti-cancer activity assay.

Macrophage cell lines (RAW 264.7) were placed into wells of a 96-well microtiter plate (2.5×10^5 per well) and were treated with 20 μ l of CBF (L15, L10, L21 and L23) or *L. edodes* culture medium for 48 h. After incubation, culture supernatant was collected by centrifugation and then added (20 μ l/well) to MRC-5, Hela, AGS, Hep G2 and MCF-7 contained in 96-well microtiter plates. The entry with *L. edodes* culture medium was used as control that contained only the cells. The plates with stimulated cells were incubated for 72 h at 37°C. The MTT solution prepared in PBS at 5 mg/ml concentration was added (20 μ l) to each well. Culture macrophage medium is the control. Further analysis was carried out as described in anti-cancer activity for MTT colorimetric assay.



The resulting data of all the above experiments are presented as a mean (S.D. from triplicates).

Cell-cell communication assay.

Macrophage cell lines were placed into wells of a plate (2.5×10^5 cells/ml) and were treated separately with 100 μ l of PBS as a control, LPS (10 μ g/ml) as a positive control, and L15 polysaccharides (1.5 mg/ml) as test sample. They were then incubated for 24 h at 37°C. Cell-cell communication of macrophage was measured

applying the scrape loading dye transfer technique. The fluorescent dye Lucifer yellow (Sigma), Dissolved in PBS, was added the cells (RAW 264.7). After incubation the cells were washed with PBS to remove extracellular dye. Dye migration was observed and digitally photographed at 200× using Nikon eclipse TE 2000-s microscope.

Pressurized water extraction.

The crude polysaccharides were then treated with pressurized water (ISCO, 260D) at different pressures and time periods to obtain the PWE products. Normally, 35 mg crude polysaccharides were added to each extraction cell (10 ml). The effects of the two factors, i.e. pressure and time, were investigated in the ranges of 2.5-25.3 MPa and 10-80 min at 28°C. The PWE polysaccharides were filtered (Milli-pore, 0.22 µm) for immunoassay and the residues were dried to determine recovery yields. The BWE polysaccharides obtained at 0.1 MPa for 40 min were used for control experiments (81). The data of the above experiments were presented as a mean and relative standard deviation (R.S.D. %) of three replicate measurements.

Macrophage morphology.

Macrophage cell lines were placed into wells of a microtiter plate (2.5×10^5 cells/ml) and were treated separately with 100 µl of PBS (10 µg/ml) as a blank,

lipopolysaccharide (LPS, 10 µg/ml, Sigma) as a positive control, and BWE and PWE polysaccharides (10.1 MPa, 70 min). They were then incubated for 24 h at 37°C. After decanting the supernatant, a cell culture medium (DMEM) was added at the end of incubation. The macrophage morphologies were observed utilizing differential interference contrast (DIC) microscopy (Leica DMRXA, photo CCD-Leica DC500).

Multiple linear regression analysis (MLRA).

Multiple linear regression analysis was conducted similarly to a previous reported paper with minor modifications (58). With a multiple regression model, the relationship is described using a generalization of a straight line equation (equation 1):

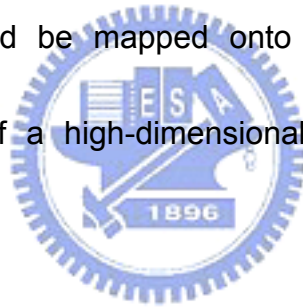
$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \dots + \beta_p X_p + e \quad (1)$$

where Y denotes the response variable (NBT reduction value), β_0 is a constant (intercept), X_1, X_2, \dots, X_p is a vector of p predictor variables (monosaccharide composition value), $\beta_1, \beta_2, \dots, \beta_p$ is the vector of p regression coefficients. Each predictor variable has its own coefficient, and the outcome variable is predicted from a combination of all the variables multiplied by their respective coefficients plus an error term. The relationship was determined using the least square fitting technique. A residual analysis was applied to check the model fitting (82). MLRA was performed utilizing the computer software-Statistical Package for the Social Sciences (SPSS)

and S-PLUS.

Principal component analysis (PCA).

Principal component analysis was used to transform a number of potentially correlated variables (descriptors) into a number of relatively independent variables that could be ranked based upon their contribution for explaining the variation of the whole data set (59). Based on this method, the relatively important components of high-dimensional pattern could be successfully identified. Thus, the original high-dimensional data could be mapped onto a lower dimensional space, and therefore the complexity of a high-dimensional pattern classification problem is substantially reduced (54).



For a random vector \mathbf{x} , where

$$\mathbf{x} = (x_1, \dots, x_n)^T \tag{2}$$

the mean of the random vector is denoted by

$$\mu_{\mathbf{x}} = E\{\mathbf{x}\} \tag{3}$$

and the covariance matrix of the same random vector is

$$\mathbf{C}_{\mathbf{x}} = E\{(\mathbf{x} - \mu_{\mathbf{x}})(\mathbf{x} - \mu_{\mathbf{x}})^T\} \tag{4}$$

The components of $\mathbf{C}_{\mathbf{x}}$, denoted by C_{ij} , represent the covariances between the random variable components x_i and x_j . The component C_{ii} is the variance of the

component x_i . The variance of a component indicates a measure of spread of the component values around its mean value. If two components x_i and x_j of the data are uncorrelated, then their covariance is zero ($C_{ij} = C_{ji}=0$). The covariance matrix is, by definition, always symmetric. From a sample of vectors X_1, \dots, X_M , the sample mean and sample covariance matrix can be calculated to estimate the mean and the covariance matrix respectively.

From a symmetric matrix such as the covariance matrix, an orthogonal basis can be calculated by finding its eigenvalues and eigenvectors. The eigenvectors e_i and the corresponding eigenvalues λ_i are the solutions of the equation (83):

$$C_x e_i = \lambda_i e_i, \quad i=1, \dots, n \quad (5)$$

For our present study, data matrix consisted of the monosaccharide compositions of ten strains of *L. edodes* and pattern recognition based on PCA was performed using the SPSS (59, 62, 82).

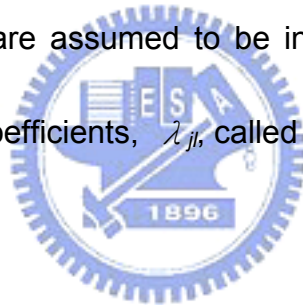
Factor analysis (FA).

Factor analysis was applied to describe the correlation among several variables in terms of a few quantities, i.e. factors. FA and PCA are two different approaches to analyze the correlation structure of a set of real valued random variables. In practice, it is often observed that the results of FA are very close to those of PCA. These two

approaches are strongly related to each other (84). FA uses an estimate of common variance among the original variables in order to generate the factor solution. A factor is the linear combination of original variables. The number of factors will always be less than the number of original variables (59). The aim of FA is to summarize the correlation structure of observed variables X_1, X_2, \dots, X_p . For this purpose one constructs $k < p$ unobservable or latent variables f_1, \dots, f_k , which are called the factors, and which are linked with the original variables through the equation for each $1 \leq j \leq p$.

$$X_j = \lambda_{j1}f_1 + \lambda_{j2}f_2 + \dots + \lambda_{jk}f_k + \varepsilon_j \quad (6)$$

The error variables $\varepsilon_1, \dots, \varepsilon_p$ are assumed to be independent, but they have specific variances ψ_1, \dots, ψ_p . The coefficients, λ_{j1} , called factor loadings, are elements of the matrix of loadings Λ (85).



Factor analyses to evaluate relationships between different strains and monosaccharide compositions were also carried out using SAS (59, 86).

Cluster analysis.

Cluster analysis methods were applied to investigate the relationships between types of glycosyl linkages (using the relative GC-MS abundance data) and *L. edodes* strains. The relative abundances of all glycosyl linkages in the ten samples were normalized to the myo-inositol internal standard of the L1 sample and were used for

later cluster analysis (59). Two key steps are involved in cluster analysis. The first is the measurement of the object similarity based on the Euclidean distance or Manhattan distance between objects (67). The second is to group the objects based upon the resulted distances (linkages). Linkage methods are based upon how the association between groups is measured.

The Euclidean distance ($D_{Eucl.}$) between any two objects x_i and y_i was probably the most commonly chosen type of distance and in the multidimensional space was computed as below:

$$D_{Eucl.}(x,y) = \{\sum (x_i - y_i)^2\}^{1/2} \quad (1)$$

This method has certain advantages, e.g., the distance between any two objects is not affected by the addition of new objects to the analysis, which may be outliers.

The Manhattan distance ($D_{Mann.}$) is simply the average differences across dimensions. In most cases, the results of these distance measurements are similar to those of simple Euclidean distances (59, 87). However, the effect of single large differences (outliers) is dampened (since they are not squared) in this measure. The Manhattan distance is computed as below:

$$D_{Mann.}(x,y) = \sum |x_i - y_i| \quad (2)$$

In this study, both Euclidean and Manhattan distances with five clustering methods were employed: average, complete, single, Ward's and weighted linkage

type (87). Further standardization (i.e. Z-score normalization) of variables has been undertaken to enable the comparison of variables to minimize the bias in weighting which may result from differing measurement scales and ranges (59). Then, the linkage type similarity within each cluster was calculated and plotted. The analysis was performed using S-PLUS.



PART I

Polysaccharide Composition, Structure, *In Vitro* Immuno-Modulating and Anti-tumor Activities of Some Regionally Different Strains of *Lentinula edodes*

Abstract

The monosaccharide composition, molecular mass, structural linkage, immuno-modulating and anti-tumor activities were investigated for polysaccharides extracted from different phylogenetic groups of ten regional *L. edodes*. The immuno-modulating properties and anti-tumor activities of these *L. edodes* extracts including crude polysaccharides and hot-water-extracts obtained from culture broth filtrates were tested using several different cell lines. The results showed that the ten isolated *L. edodes* could be classified into three distinct groups using amplified fragment length polymorphism assay. All isolates had similar molecular mass distribution between 1×10^2 and 3×10^3 kDa and the monosaccharide composition analysis revealed the presence of glucose, mannose, xylose, galactose, fucose, rhamnose and arabinose in different ratios. The elucidated structural features of a crude polysaccharide fraction showed a new form of polysaccharide linkage with a backbone of α -(1→4)-glucan and side chains of β -(1→6)-glucan. Most of the culture

broth filtrates exhibited significant enhancement in macrophage stimulatory activity. Indirect in vitro anti-cancer assay was also positive indicating that the effect is possibly due to a preliminary macrophage stimulatory activity with subsequent release of TNF- α or other biochemicals to inhibit tumor cell growth.

Results and discussion

The step-wise experimental procedures adopted to prepare culture broth filtrate (CBF) and to isolate boiling water extract (BWE) of crude polysaccharides for composition analysis and anti-cancer activity assays are shown in Fig. 1.



***L. edodes* culture.**

The optimum environmental conditions for culture growth and polysaccharides production of mushrooms in liquid cultures are dependent on strains. Initially, the growth conditions of the culture were optimized that showed a stationary phase after 14 days. It was observed that *L. edodes* produced most polysaccharides and showed highest immuno-stimulating activity in the stationary phase. The biomass of the CBF extracts of all strains was estimated to be in the range 0.35 to 0.40 g/50 ml and pH between 2.5 and 2.8 for 14 days (Fig. 2 and Fig. 3). These values would be useful as references for optimization and pilot plant production studies in the future. Recently,

the submerged cultivation of mushroom has received much attention in Asian regions as a promising alternative for efficient production of its valuable metabolites, especially polysaccharides and ganoderic acids (38, 88). It usually takes several months to cultivate the fruiting body of the mushroom, and it is also difficult to control product quality during soil cultivation. There is a great need to supply the market with a large amount of high-quality mushroom products. Therefore, submerged cultivation of mushroom could eventually supplement the need and proves useful over fruiting body cultivation.

Strain typing/phylogenetic mapping.



The ten isolates of *L. edodes* were grouped into three distinct clusters by AFLP: (1) L24 and L25 isolates from China; (2) L1 and L4 isolates from Taiwan; (3) L6, L10, L11, L15, L21 and L23 isolates from Japan. The DNA material and adaptors were digested with restriction enzymes, *Mse* I and *Eco* RI, to prepare the AFLP template for sequence analysis. Two primer sets, *Eco* RI-AC-FAM/*Mse* I-CAA and *Eco* RI-AA-FAM/*Mse* I-CAC, were employed for selective amplification. The band positions of selected primers were used to construct a similarity index and an attempt was made to match the regionally different strains. A dendrogram of the similarity index based on the bands obtained for selected two primer sets was plotted to

distinguish the closely related strains (Fig. 4). A very close genetic homogeneity among cultivated strains of Japanese mushrooms, L11, L15 and L10 was seen. Similarly, for the Taiwanese and Chinese mushrooms, the obtained AFLP fingerprints point out close resemblance to the genetic homogeneity. However, L24 and L25 mushrooms from China were quite different from the Japanese and Taiwanese mushrooms. The mushrooms, L1 and L4 were cultivated heterogeneous strains comprising of Japanese strains SL-19 and 271.

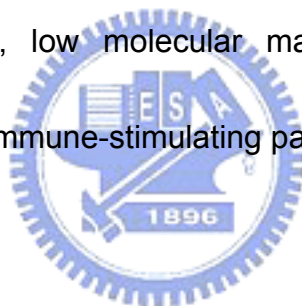
The dendrogram obtained using AFLP (fingerprinting) analysis of *L. edodes* provides new insight into the population structure of this mushroom species and proves useful for phylogenetic type studies. The results appear promising and are well supported by results obtained using random amplified polymorphic DNA (RAPD) assay (fingerprinting) for the same species (89). Therefore, application of AFLP fingerprint assay for phylogenetic studies of mushrooms can now be included among other reported species in population studies.

Immuno-modulating and anti-cancer activities.

The molecular mass fractions and chemical compositions of polysaccharides produced are strongly dependent on strain variations, extraction methods and culture conditions (56). Therefore, a comparative study of immuno-modulating and

anti-cancer properties using CBF deserved further investigation. The CBF of all mushroom strains were used to treat macrophage (RAW 264.7 cell line) to test the immuno-stimulating activity (Fig. 5A). The strains with increasing NBT % reduction follow the order: (L15>L23>L10)> (L21>L6)> (L11> L24)> (L1>L25>L4). Based on the order of highest immuno-stimulating activity exhibits, the CBF of L15 and L23 strains were further tested for its ability to produce TNF- α (Fig. 5B). When lipo-polysaccharide or glucan extracts alone were used, TNF- α release was not impressive. But adding them together resulted in synergistically increased production of TNF- α . Similar behavior was observed previously among different strains of fungi and it was suggested that lipo-polysaccharide triggers a so-called 'priming effect' on polysaccharides (40). Thus, it was necessary to conduct further indirect anti-cancer activity experiments using CBF. Indirect anti-cancer activity tests were conducted on all the selected cancer cell lines with CBF of L15, L23, L10 and L21 strains (Fig. 5C). The cancer cell survival rate was moderate (<60% for AGS and MCF-7) with L15 and very high with L23 (for all selected cancer cell lines). Direct anti-cancer activity experimental results did not show effective inhibition of tumors (Fig. 5D). Among all the cell lines, gastric cancer cell line (AGS) responded to some extent better than others. Normal cell line (MRC-5) responded in a similar range to all other cancer cell lines.

Both CBF and BWE of L15 strain show better performance in tests for immuno-modulating properties. The BWE of L15 was further fractionated to obtain four different molecular mass fractions (Fig. 6). The polysaccharide content of the fractions was in the range 0.2-0.7 mg/ml (Fig. 7A). They were further tested for immuno-stimulating activity and roles in the direct anticancer activity. The immunity-enhancing properties of BWE's were similar to those of CBF. The results of immuno-stimulating activity assays of mixtures of all fractions were similar to individual high molecular mass fractions 'B' (Fig. 7B, Fig. 8). However, in the direct anti-cancer activity assays, low molecular mass fractions gave better results confirming their vital role in immune-stimulating pathways (Fig. 7C).



Cell-cell communication assay.

The scrape loading/dye transfer were used to demonstrate the macrophage cellular communication. The phase contrast and fluorescent images (Fig. 9A) shows the normal macrophage unable to transfer dye to neighboring cells. The LPS or L15 polysaccharides-treated macrophage, the results shows the lack of Lucifer yellow dye transfer to cells (Fig. 9B and Fig. 9C). The macrophage only treated with L15 polysaccharides or LPS can not exhibition cell-cell communication. Interestingly, an additive effect of the macrophage first treated with L15 polysaccharides and then

treated with LPS on dye transfer was found in Fig. 9D. The Fig. 9D (L15 polysaccharides plus LPS) shows the dye transfer ability higher than the Fig. 9E (LPS plus L15 polysaccharides).

Molecular mass and monosaccharide composition.

To deduce the structure-activity relationship, it is important to determine the molecular mass distribution and monosaccharide composition of the various polysaccharides isolates. Gel permeation chromatographic studies showed that all isolated polysaccharides had up to four similar molecular mass fractions (designated as A, B, C, D) with different distribution in the range 1×10^2 kDa and 3×10^3 kDa (Fig. 10). A thorough examination revealed that the molecular mass fractions A and D were present in all strains while fractions B and C showed some disparity.

The monosaccharide composition was determined from the standard calibration curves plotted for individual monosaccharide. The data revealed differences in the distribution of glucose, mannose, xylose, galactose, fucose, rhamnose and arabinose in the CBF of all mushrooms (Table 1). For example, the L15 polysaccharides contained mainly glucose and mannose and the contents of glucose in PS of L1, L4, L6, L10, L11, L21, L23, L24 and L25 were 55.4%, 23.1%, 51%, 56.2%, 47.1%, 47.4%, 56.4%, 88.8% and 55.6%, respectively. The nature of these compositions and

differences remain unclear at this moment and would need further investigation when more relevant knowledge and analytical skills are available.

Structures.

The structural features of the crude polysaccharides/BWE extracted from strain L15 were elucidated by using the FT-IR, NMR and GC-MS spectroscopic techniques to establish structure-immuno-stimulating and anti-cancer activity relationships. In the FT-IR spectra of the crude PS (all strains), the bands corresponding to the ν (C=O) vibration in the carboxyl group at 1650 cm^{-1} indicate that this carboxyl group was hydrogen bonded (Fig. 11). The absence of carbonyl bands at 1535 cm^{-1} and 1700 cm^{-1} indicates that these strains contain neither proteins nor uronic acids, respectively. In addition to the characteristic bands of glucans in the $1000\text{-}1100\text{ cm}^{-1}$ range, FT-IR spectra showed a weak band at 850 cm^{-1} (Fig. 11) that revealed the ' α ' configuration of the main glucan linkages (79).

The chemical shifts of individual proton and carbon peaks are shown in the NMR spectra of L15 BWE (Fig. 12). The two groups of anomeric proton signals centered at δ 5.29 and 4.93 ppm were assigned to (1 \rightarrow 4)-D-Glcp and (1 \rightarrow 6)-D-Glcp, respectively, Fig. 12A (71). The two major carbon peaks at δ 99.68 and 102.43 ppm were assigned to α -(1 \rightarrow 4)-D-Glcp and β -(1 \rightarrow 6)-D-Glcp residues, respectively (Fig. 12B). The


anomeric carbon signals at δ 71.55, 71.28, 76.06, 70.09, and 60.86 ppm were assigned, respectively, to C-2, C-3, C-4, C-5, and C-6 of α -(1 \rightarrow 4)-D-Glcp, due to their relatively higher peak intensities. The other carbon signals assigned for β -(1 \rightarrow 6)-D-Glcp are C-2 (73.16), C-3 (76.79), C-4 (69.75), C-5 (73.45) and C6 (66.96). Further confirmation was based on analysis for alditol acetates using GC-MS in crude polysaccharides of L15 (Fig. 13). The resulting chromatograms demonstrated the presence of α -(1 \rightarrow 4)-linked-D-glucopyranosyl and β -(1 \rightarrow 6)-linked-D-glucopyranosyl moieties in the glucan, i.e. peak a: 1,4,5-tri-O-acetyl-1-deuterio-2,3,6-tri-O-methyl-D-glucitol (m/z: 43, 59, 71, 87, 102, 118, 129, 142, 162, 173, 233; α -(1 \rightarrow 4)) and peak b: 1,5,6-tri-O-acetyl-1-deuterio-2,3,4-tri-O-methyl-D-glucitol (m/z: 43, 59, 71, 87, 102, 118, 129, 143, 162, 173, 189, 233; β -(1 \rightarrow 6)). The 1,4,5,6-tetra-O-acetyl-1-deuterio-2,3-di-O-methyl-glucitol (m/z: 43, 59, 74, 85, 102, 118, 127, 142, 162, 201, 261) indicates (1 \rightarrow 4) and (1 \rightarrow 6) linkage (71).

Linkage assignment was further confirmed by the ^1H - ^{13}C HSQC 2D-NMR spectrum that shows the cross relationships of the proton and carbon peaks that define glycosidic linkages (71, 90, Fig. 14). Note that the chemical shifts of C-4 and C'6 typify (1 \rightarrow 4)-linked and (1 \rightarrow 6)-linked glucose residues, respectively (91). Also note that the relative positions of the anomeric signals for both constituents (denoted H-1 and H'-1) are typical for an α -anomeric and a β -anomeric configuration. Although

this linkage has been found for other mushrooms, it is new for the BWE from *L. edodes*.

Analysis of the L15 BWE monosaccharide composition revealed that the one with the highest content is glucose (68.9%), the next is mannose (20%), and the remaining five constitute a total of 11%. This composition is rare, particularly for the presence of a total of seven different monosaccharides, and has not been reported in the literature. The tentative ^{13}C NMR peak assignments for the mannopyranosyl residual are: C1 (96.02), C2 (69.65), C3 (70.35), C4 (78.5), C5 (74.25), C6 (61.2) (37, 92).

Discussion



The procedures adopted to test the immuno-modulating and anti-cancer activities were similar to the experiments described by several authors (34, 80). The common protocol accepted is the use of fruiting bodies, extractions using aqueous or non-aqueous phases and structural characterization of the isolated products. Some experimental modifications were introduced including the method of isolation of the polysaccharides and the experimental steps involving treatment of cancer cell lines with polysaccharides. An important result of this study was the ability of the polysaccharides to stimulate immune cells irrespective of method of isolation, i.e. either using CBF or BWE or fractionated BWE. The polysaccharides from both BWE

and crude polysaccharide seem to contain similar backbone structures.

Ohno *et al.* (40) reported that the release of TNF- α by macrophages could be induced by β -glucans with specific molecular weights and lower branching ratios. The mechanisms for the recognition of β -glucans by macrophage were proposed to be fairly complex and the β -glucans were assumed to be broken down to lower molecular weight fragments through various cellular functions (40, 43). Also, the addition of lipo-polysaccharide resulted in a “priming effect” and increased the TNF- α production by various β -glucans (40, 93). Our results showed that structurally different α -glucans extracted from different mushroom strains could also stimulate RAW 264.7 cells to secrete TNF- α . A synergistic effect on the TNF- α release by adding lipo-polysaccharide was also observed in the *in vitro* studies. It would be of interest to see if the same effect is observed in the *in vivo* studies.

In the indirect anti-cancer activity, the cytotoxic response of peritoneal macrophages and enhanced release of TNF- α are accounted for increased immuno-stimulating activity. Among the regional strains classified, the mushroom isolates from Japan showed the highest immuno-stimulating activity, followed by isolates from China and Taiwan, respectively. polysaccharides in different strains of mushroom have anti-cancer activities that differ greatly in their chemical compositions and configurations. Although it is difficult to correlate the structure and anti-cancer

activity, particularly due to the difficulty in determining the three dimensional structures, some correlations can be inferred.

Earlier investigations revealed that the enhanced immuno-stimulating activity of the mushrooms might be due to the presence of α -glucans (94). Not only mushrooms, α -glucan in the polysaccharides was also identified in several medicinal plant extracts (94-96). All of these active polysaccharides, regardless of their origin, were obtained by different extraction methods and collected at different fractions and were found to vary in polymer structures. In some of the studies, extracted glucans with varying substituted monosaccharides in the side chain have shown enhanced immuno-modulating activity (75), e.g. *Bao et al.* employing hot water extracted polysaccharides from *Ganoderma lucidum*. (73). *Ukawa et al.* found out that the neutral monosaccharide composition, i.e. fucose, xylose., mannose., galactose, and glucose, in the polysaccharides from isolates of fruiting bodies of *Lyophyllum decastes Sing.* were different from other species and this along with various protein content exerted increased anti-cancer activity (97). *Tomati et al.* (77) discovered that the proportion of the monosaccharides present in the hot water extract of CBF of *L. edodes*, glucose content was the highest and xylose second, with a 7:1 ratio. Other monosaccharides were ribose, arabinose and mannose and the total content was less than 1% (77). Similar phenomena were observed for some medicinal plant extracts,

used as anti-cancer compounds (98).

Conclusions

Natural polysaccharides with various monosaccharide compositions are difficult to synthesize in the laboratory, yet they are quite efficacious in many biological events and pharmacological treatment. We have established an experimental platform by adopting several classical and modern chemical and molecular biological protocols to prepare these materials and to use them for further studies and biotechnological applications. These include submerged liquid phase culture, separation by extraction, precipitation and chromatographic purification, molecular mass and structural characterization by various chromatographic and spectroscopic techniques such as FT-IR, NMR and GC-MS, and various biological activity assays. This report reveals that the polysaccharide fractions in the molecular mass range 1×10^2 and 3×10^3 kDa of the culture broth filtrate (CBF) and boiling water extract (BWE) from *L. edodes* mycelia are able to show macrophage-stimulating and indirect anti-cancer activities. A new form of polysaccharide linkage with a backbone of α -(1→4)-glucan and side chains of β -(1→6)-glucan has been identified. It is important to further identify the key structural features at lower molecular mass fractions necessary to maintain similar or better biological activities to establish a clearer structure-activity relationship and for more

convenient handling of future pharmacological and nutraceutical applications. The detailed mechanisms as well as related signal transductions involved with the biological activity such as macrophage-stimulation and subsequent release of TNF- α and/or other biochemicals to inhibit tumor growth need further clarification. The effects of these polysaccharides on other biological events such as cell-cell interactions are also worth exploring. We are currently in the process to do so.



Tables

Part I

Table 1. Monosaccharide composition of fractionated polysaccharide from different strains of *L. edodes*.

<i>Lentinula edodes</i>	L1	L4	L6	L10	L11	L15	L21	L23	L24	L25
Arabinose %	6.03	11.19	8.25	7.77	6.76	5.41	8.42	7.73	2.22	6.45
Xylose %	4.82	10.65	5.89	5.58	5.21	3.83	5.88	5.49	1.13	4.55
Mannose %	32.28	49.22	31.83	26.89	36.24	20.02	33.5	26.72	7.23	30.91
Galactose %	1.44	5.09	2.59	3.56	4.3	1.42	3.73	3.19	0.03	1.87
Glucose %	55.44	23.1	50.95	56.2	47.12	68.93	47.36	56.36	88.82	55.58
Rhamnose %	0	0.22	0.24	0	0.14	0.28	0.56	0.32	0.45	0.4
Fucose %	0	0.52	0.24	0	0.24	0.11	0.56	0.19	0.12	0.24
Exopolysaccharide	0.58	0.61	0.53	0.31	0.48	0.44	0.2	0.15	0.59	0.59
Content (mg/ml)										

Figure Captions

Part I

Fig. 1: Step-wise experimental protocol adopted to isolate CBF and crude polysaccharide from *L. edodes* mycelia.

Fig. 2: Time course of the mycelium growth of *L. edodes* 'L11' strain submerged liquid culture.

Fig. 3: Dry cell weight of mushroom strains.

Fig. 4: Dendrogram of *L. edodes* constructed using AFLP assay.

Fig. 5: (A) Macrophage stimulatory activity assay (% NBT reduction) using CBF of all strains. (B) TNF- α release activity using CBF of L15 and L23. (C) Indirect anti-cancer assay (% MTT reduction) using CBF of 4 different strains. (D) Direct anti-cancer assay using CBF of all strains. The CBF was first treated with immune cell lines-RAW 264.7 (% NBT reduction) and J45.01 (% MTT reduction) and then added to other different cancer cell lines (% MTT reduction).

Fig. 6: Molecular mass fractions of L15 BWE. (A) >2750 kDa (B) ~2700 kDa (C) ~534 kDa (D) ~11.7 kDa.

Fig. 7: (A) Polysaccharides content of different molecular weight fractions (A, B, C, D and E) extracted from L15 BWE and control sample from L15 BWE

mixture fractions. (B) Macrophage stimulatory activity (% NBT reduction) of obtained weight fractions, and, (C) Direct anti-cancer assay (% MTT reduction) of obtained weight fractions (PBS as control).

Fig. 8: Macrophage stimulatory activity morphological images by NBT reduction assay. (A), (B), (C), (D) and (E) show that macrophage was treated of different molecular weight fractions (A, B, C, D and E) extracted from L15 BWE (L15 BWE mixture fractions as control sample and PBS as blank control).

Fig. 9: Macrophage cell-cell communication measured by the Lucifer yellow scrape-loading/dye transfer technique. (A) Phase contrast and fluorescent images of normal macrophage, (B) LPS treatment of macrophage, (C) L15 polysaccharides treatment of macrophage, (D) L15 polysaccharides (1.5 mg/ml) plus LPS (10 μ l/ml) treatment macrophage, and (E) LPS (10 μ l/ml) plus L15 polysaccharides (1.5 mg/ml) of macrophage.

Fig. 10: GP chromatogram obtained from CBF of mushroom strains. Molecular mass fractions are indicated.

Fig. 11: A typical FTIR spectrum recorded from crude polysaccharides of L15.

Fig. 12: NMR spectra of crude polysaccharides boiling water extracted from L15. (A) The two anomeric proton signals are at δ 5.29 and 4.93 ppm that were

assigned as (1→4)-D-Glcp and (1→6)-D-Glcp (600 MHz), (B) The anomeric carbon signals for the (1→4)-D-Glcp and (1→6)-D-Glcp residues were assigned at δ 99.68 and 102.43 ppm, respectively, from ^{13}C (150 MHz). The carbon signals at δ 71.55, 71.28, 70.09, and 60.86 ppm correspond, respectively, to C-2, C-3, C-5, and C-6 of (1→4)-D-Glcp. The other signals for (1→6)-D-Glcp are C-2 (73.16), C-3 (76.79), C-4 (69.75), and C-5 (73.45),

Fig. 13: GC-MS data for the alditol acetates derived from the methylated polysaccharide BWE isolated from L15 *L. edodes*. The presence of (a) 1,4,5-tri-O acetyl-1-deuterio-2, 3, 6-tri-O-methyl-D-glucitol and, (b) 1,5,6-tri-O acetyl-1-deuterio-2, 3, 4-tri-O-methyl-D-glucitol were detected. (c) The 1,4,5,6-tetra-O-acetyl-1-deuterio-2,3-di-O-methyl-glucitol indicates (1→4) and (1→6) linkage.

Fig. 14: The 2D (HSQC) NMR spectrum of the L15 BWE in D_2O .

Part I

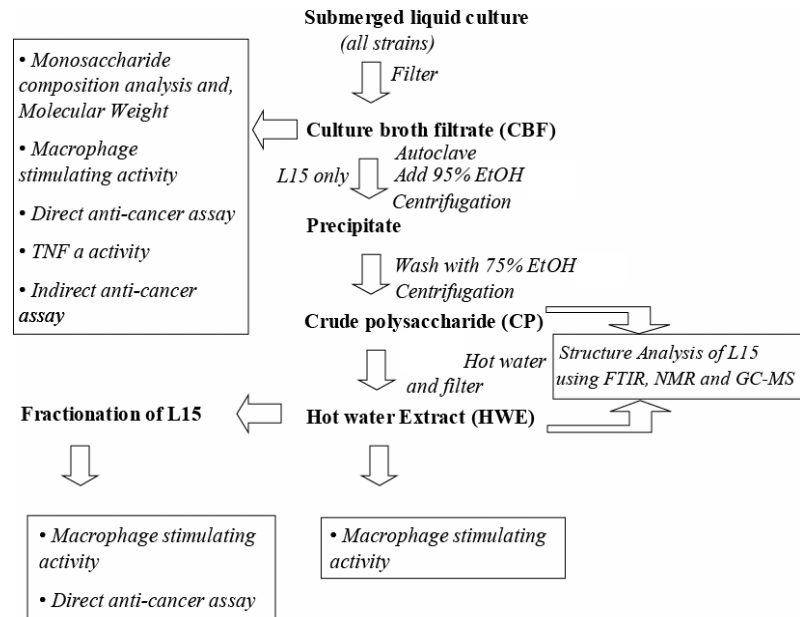


Fig. 1 Step-wise experimental protocol adopted to isolate CBF and crude polysaccharide from *L. edodes* mycelia.

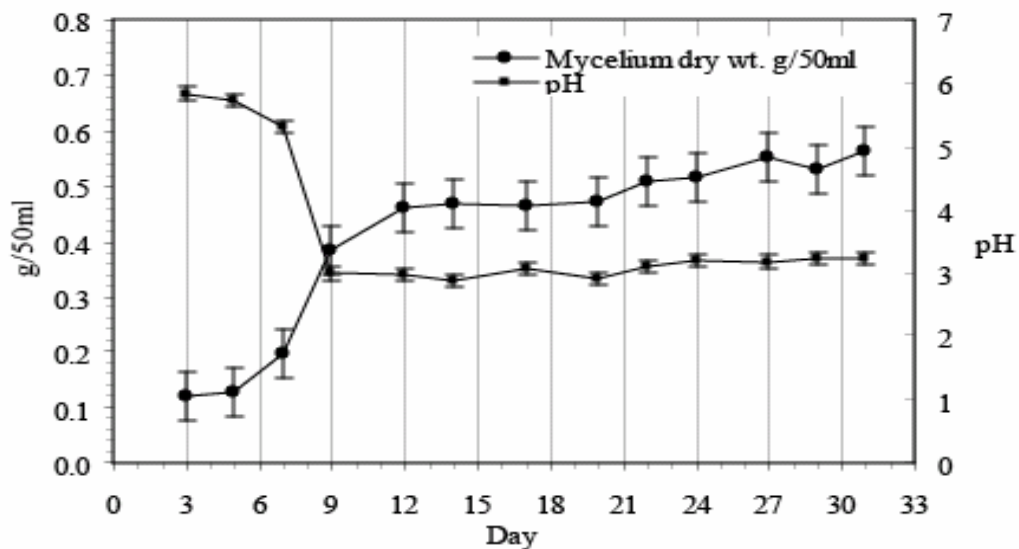


Fig. 2 Time course of the mycelium growth of *L. edodes* 'L11' strain submerged liquid culture.

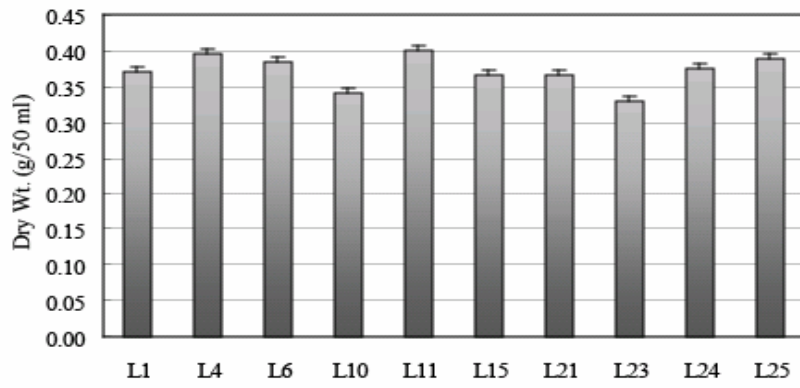


Fig. 3 Dry cell weight of mushroom strains.

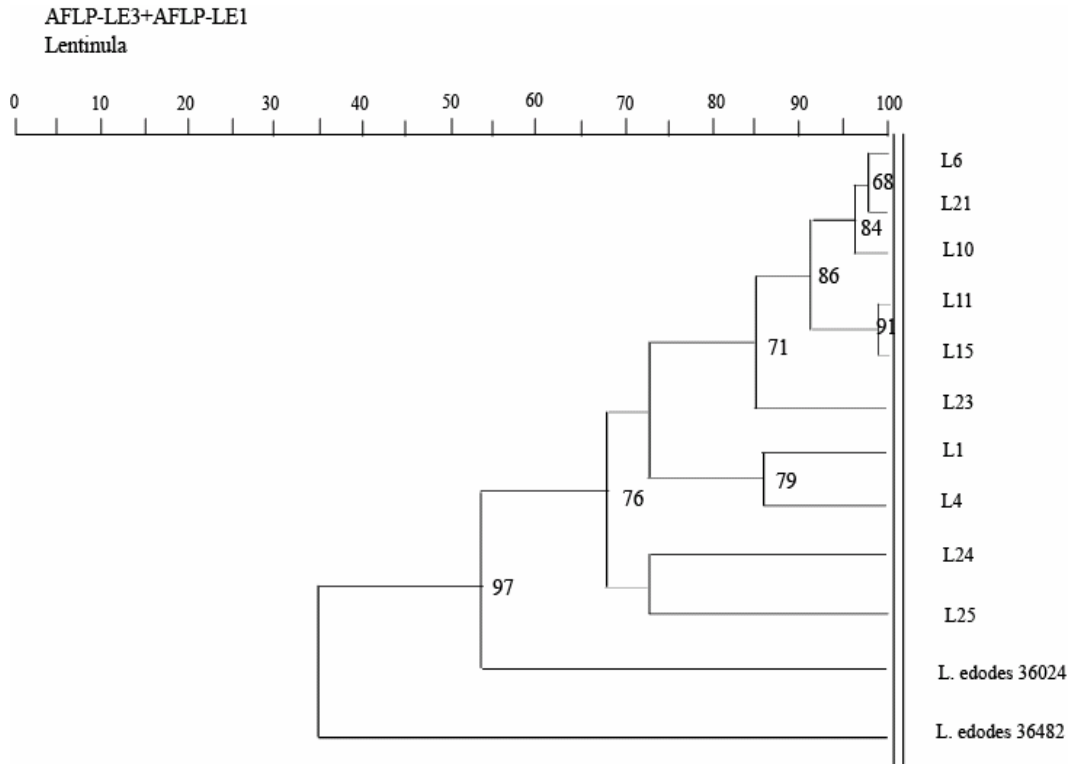
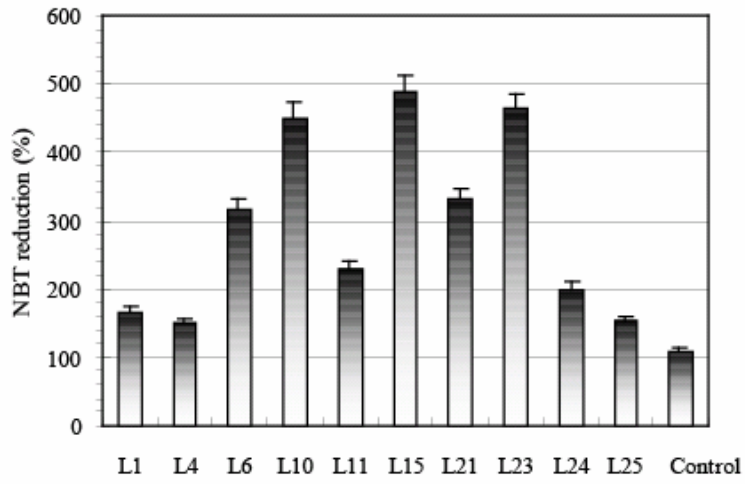
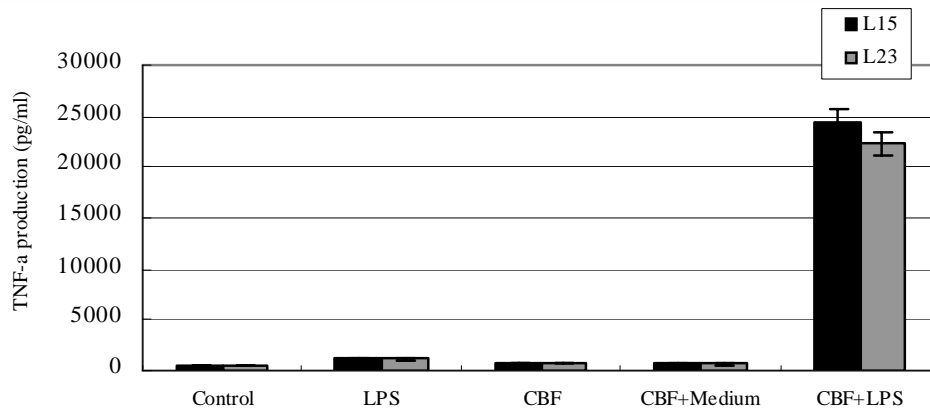


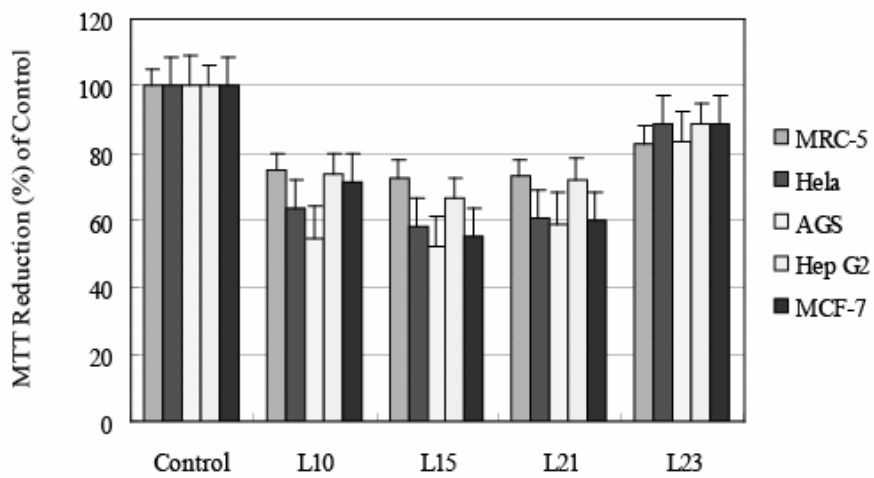
Fig. 4 Dendrogram of *L. edodes* constructed using AFLP assay.



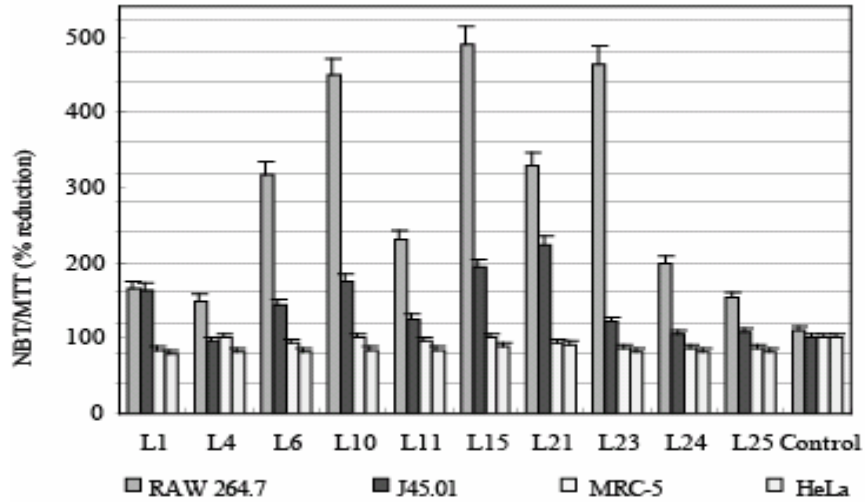
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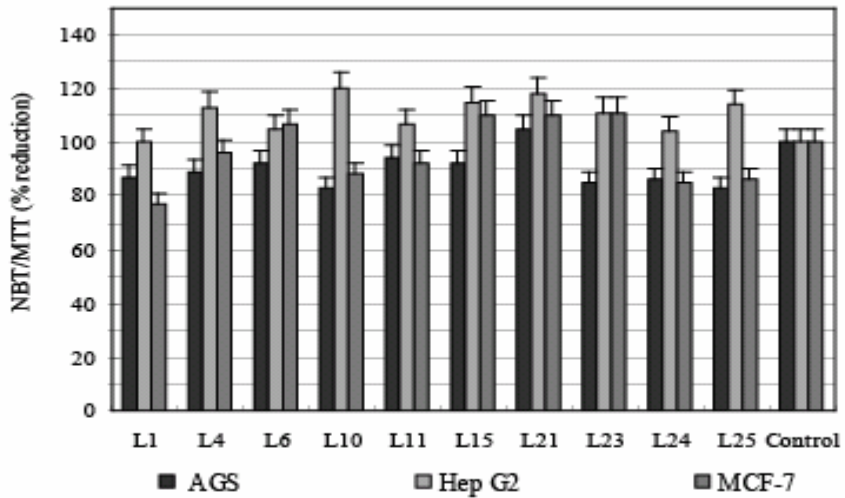
(B)



(C)



D (i)



D (ii)

Fig. 5 (A) Macrophage stimulatory activity assay (% NBT reduction) using CBF of all strains. (B) TNF- α release activity using CBF of L15 and L23. (C) Indirect anti-cancer assay (% MTT reduction) using CBF of 4 different strains. (D) Direct anti-cancer assay using CBF of all strains. The CBF was first treated with immune cell lines-RAW 264.7 (% NBT reduction) and J45.01 (% MTT reduction) and then added to other different cancer cell lines (% MTT reduction).

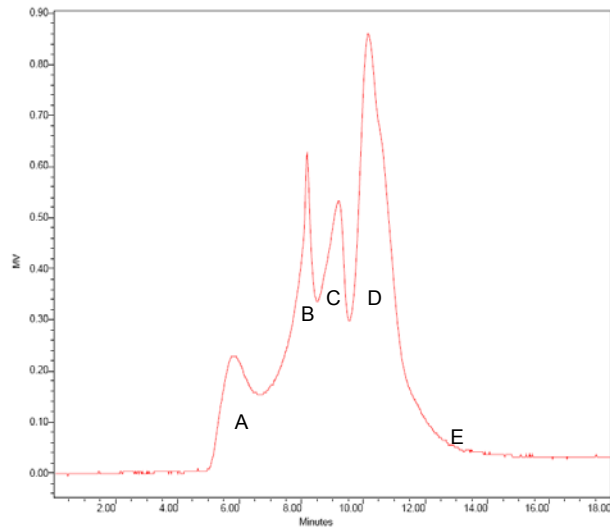


Fig. 6 Molecular mass fractions of L15 BWE. (A) >2750 kDa (B) ~2700 kDa (C) ~534 kDa (D) ~11.7 kDa.

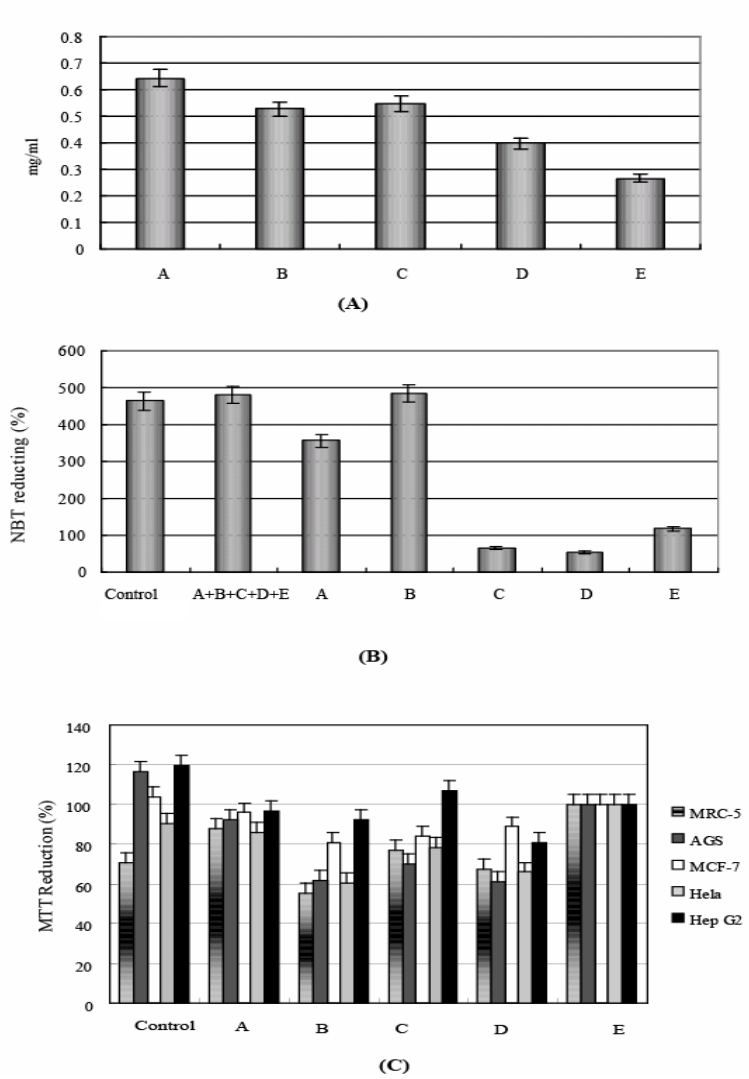


Fig. 7(A) Polysaccharides content of different molecular weight fractions (A, B, C, D

and E) extracted from L15 BWE and control sample from L15 BWE mixture fractions.

(B) Macrophage stimulatory activity (% NBT reduction) of obtained weight fractions,

and, (C) Direct anti-cancer assay (% MTT reduction) of obtained weight fractions

(PBS as control).

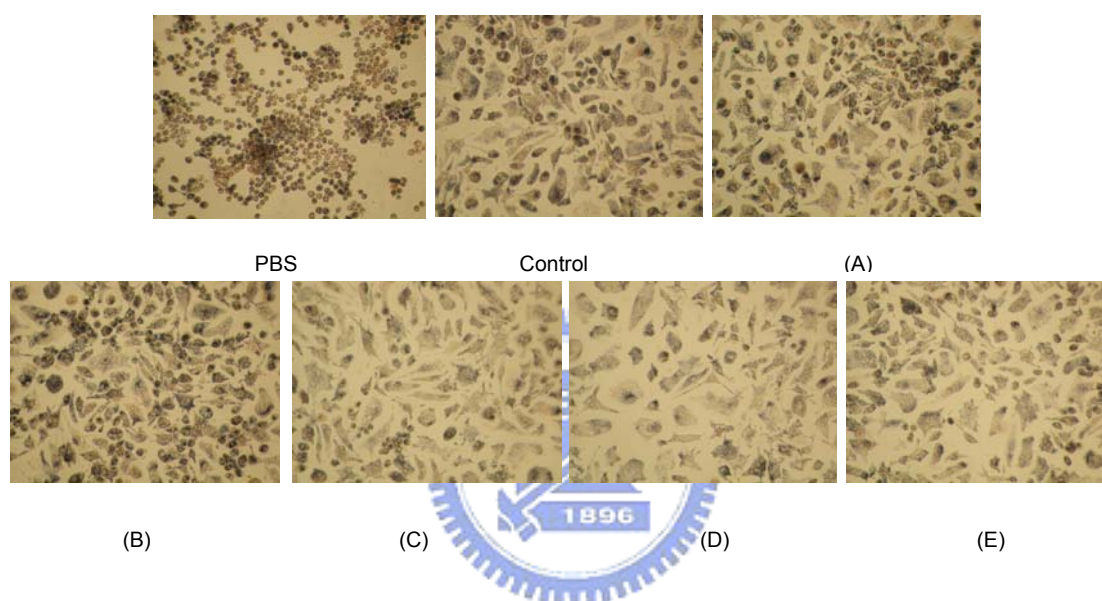


Fig. 8 Macrophage stimulatory activity morphological images by NBT reduction assay.

(A), (B), (C), (D) and (E) show that macrophage was treated of different molecular

weight fractions (A, B, C, D and E) extracted from L15 BWE (L15 BWE mixture

fractions as control sample and PBS as blank control).

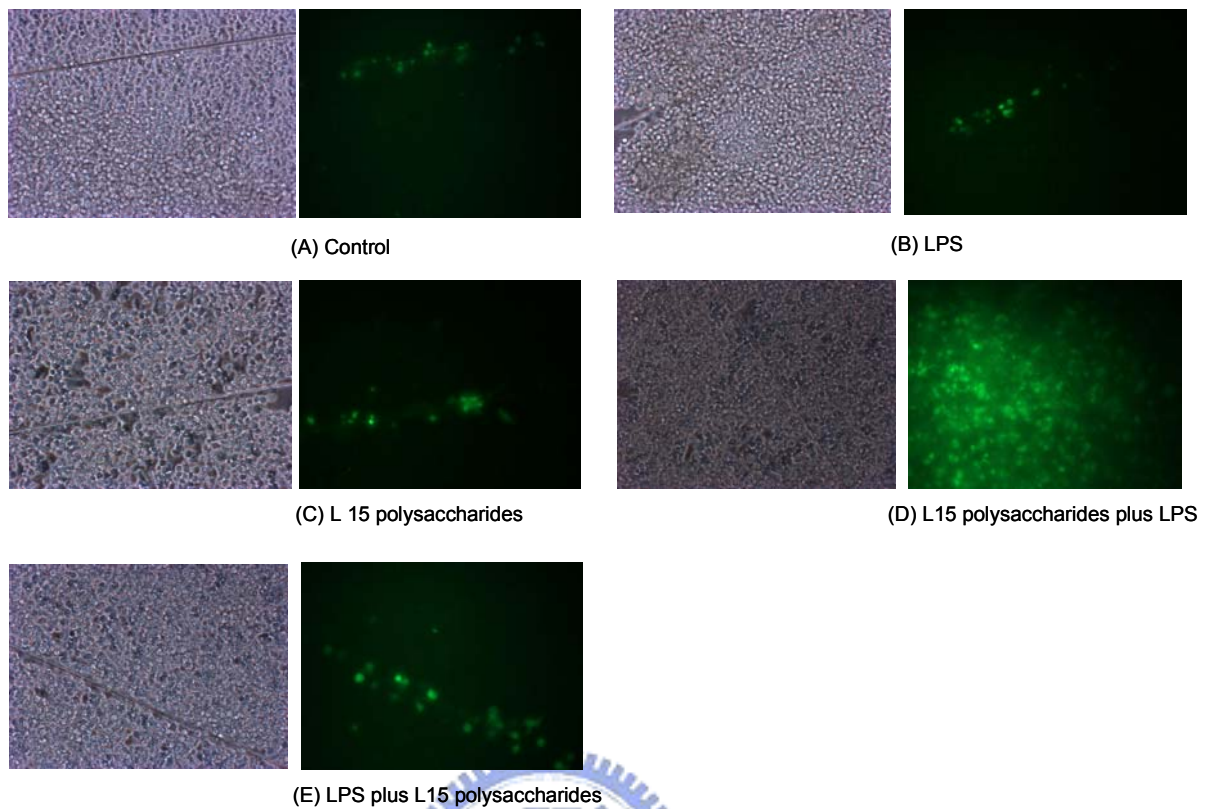


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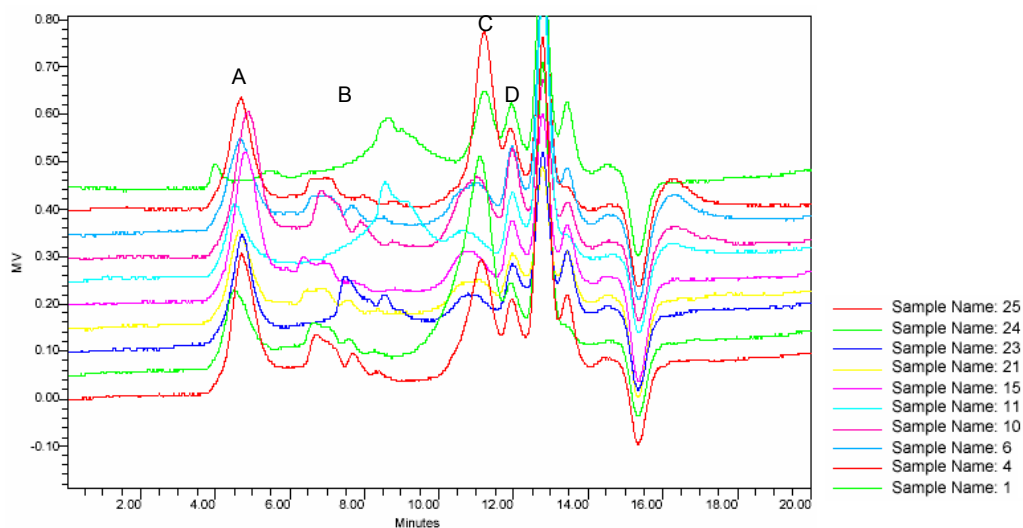


Fig. 10 GP chromatogram obtained from CBF of mushroom strains. Molecular mass fractions are indicated.

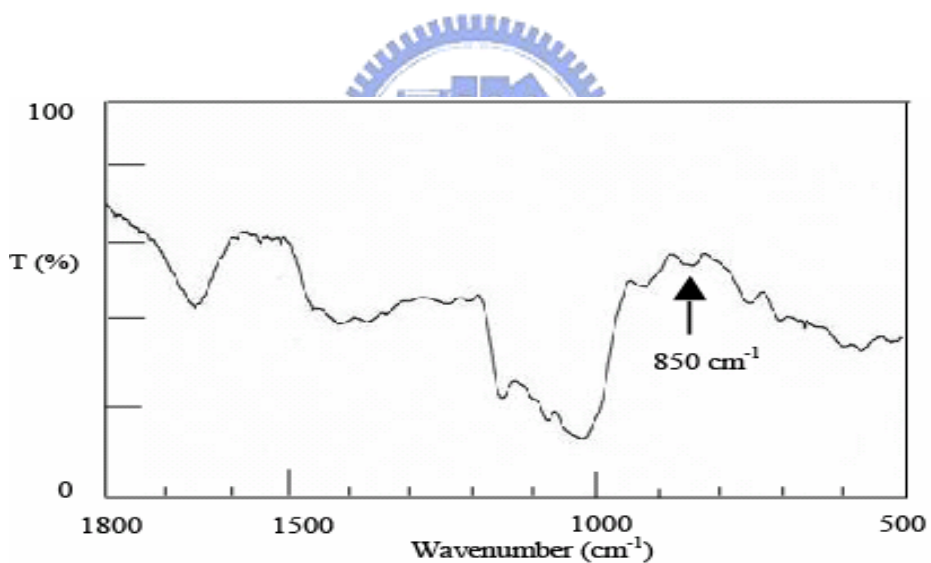
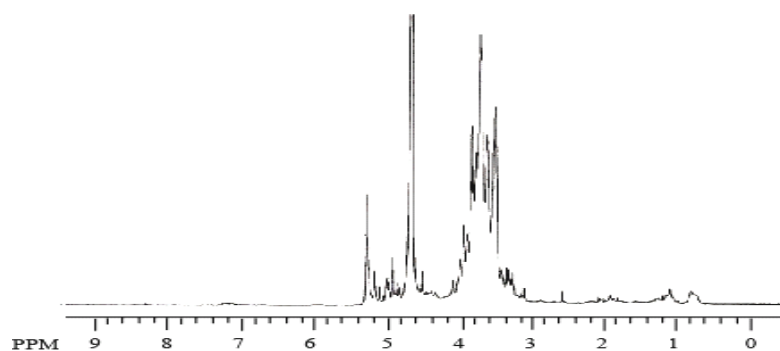
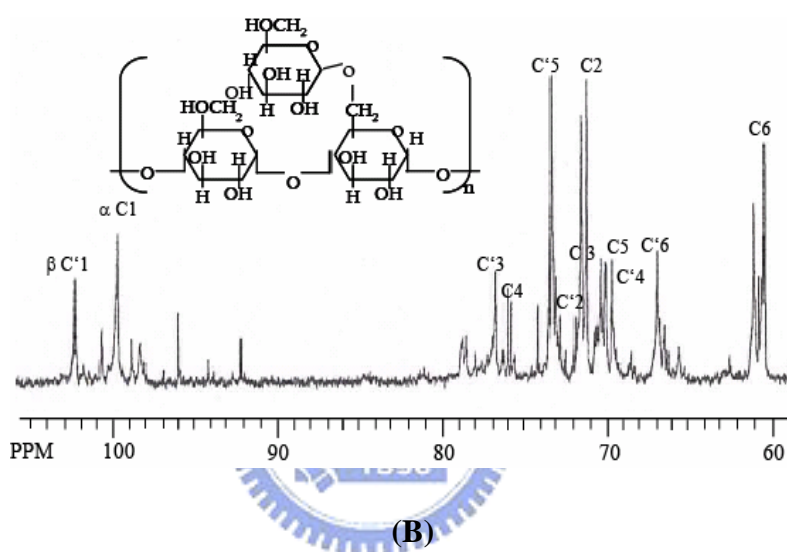


Fig. 11 A typical FTIR spectrum recorded from crude polysaccharides of L15.



(A)



(B)

Fig. 12 NMR spectra of crude polysaccharides boiling water extracted from L15. (A) The two anomeric proton signals are at δ 5.29 and 4.93 ppm that were assigned as (1 \rightarrow 4)-D-Glcp and (1 \rightarrow 6)-D-Glcp (600 MHz), (B) The anomeric carbon signals for the (1 \rightarrow 4)-D-Glcp and (1 \rightarrow 6)-D-Glcp residues were assigned at δ 99.68 and 102.43 ppm, respectively, from ^{13}C (150 MHz). The carbon signals at δ 71.55, 71.28, 70.09, and 60.86 ppm correspond, respectively, to C-2, C-3, C-5, and C-6 of (1 \rightarrow 4)-D-Glcp. The other signals for (1 \rightarrow 6)-D-Glcp are C-2 (73.16), C-3 (76.79), C-4 (69.75), and C-5 (73.45),

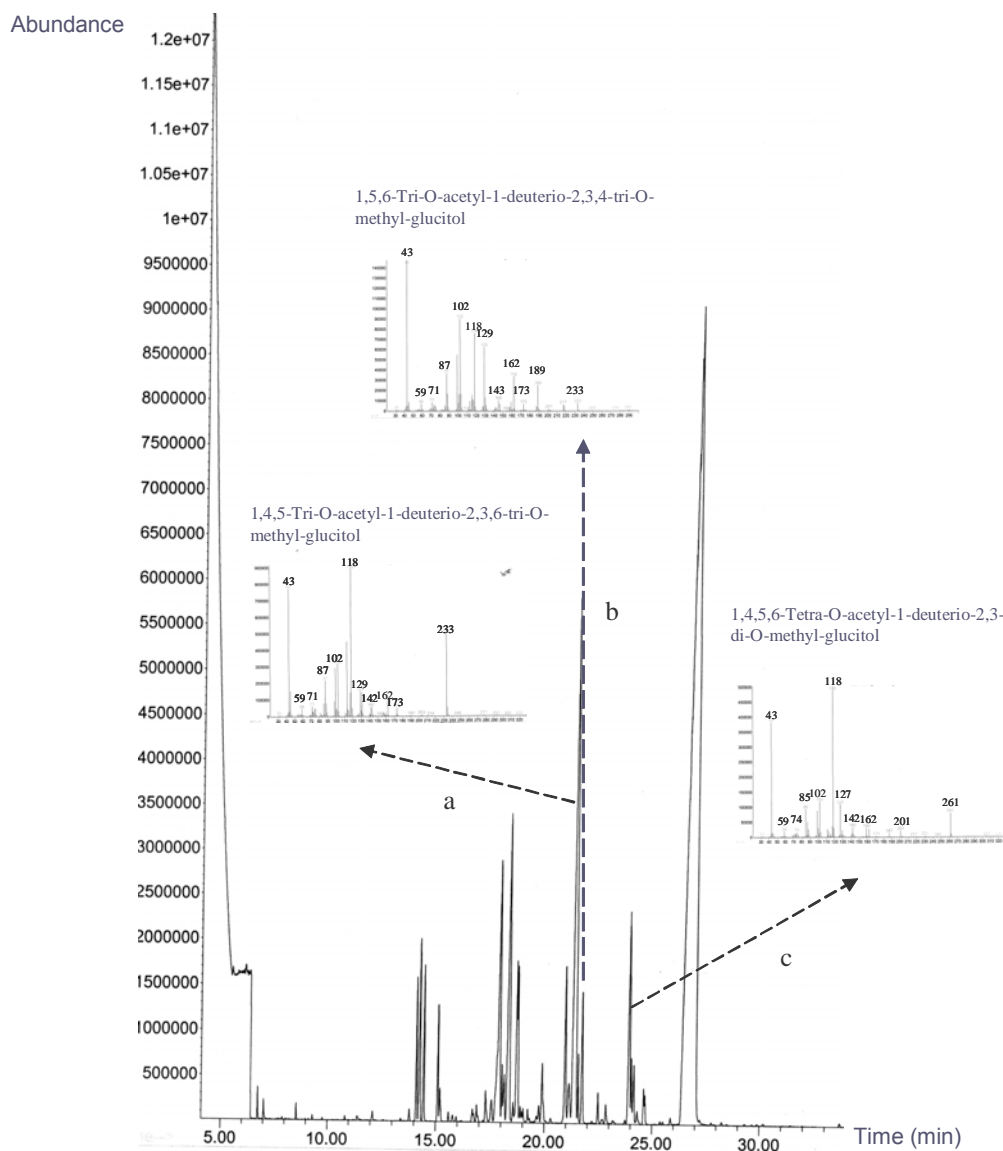


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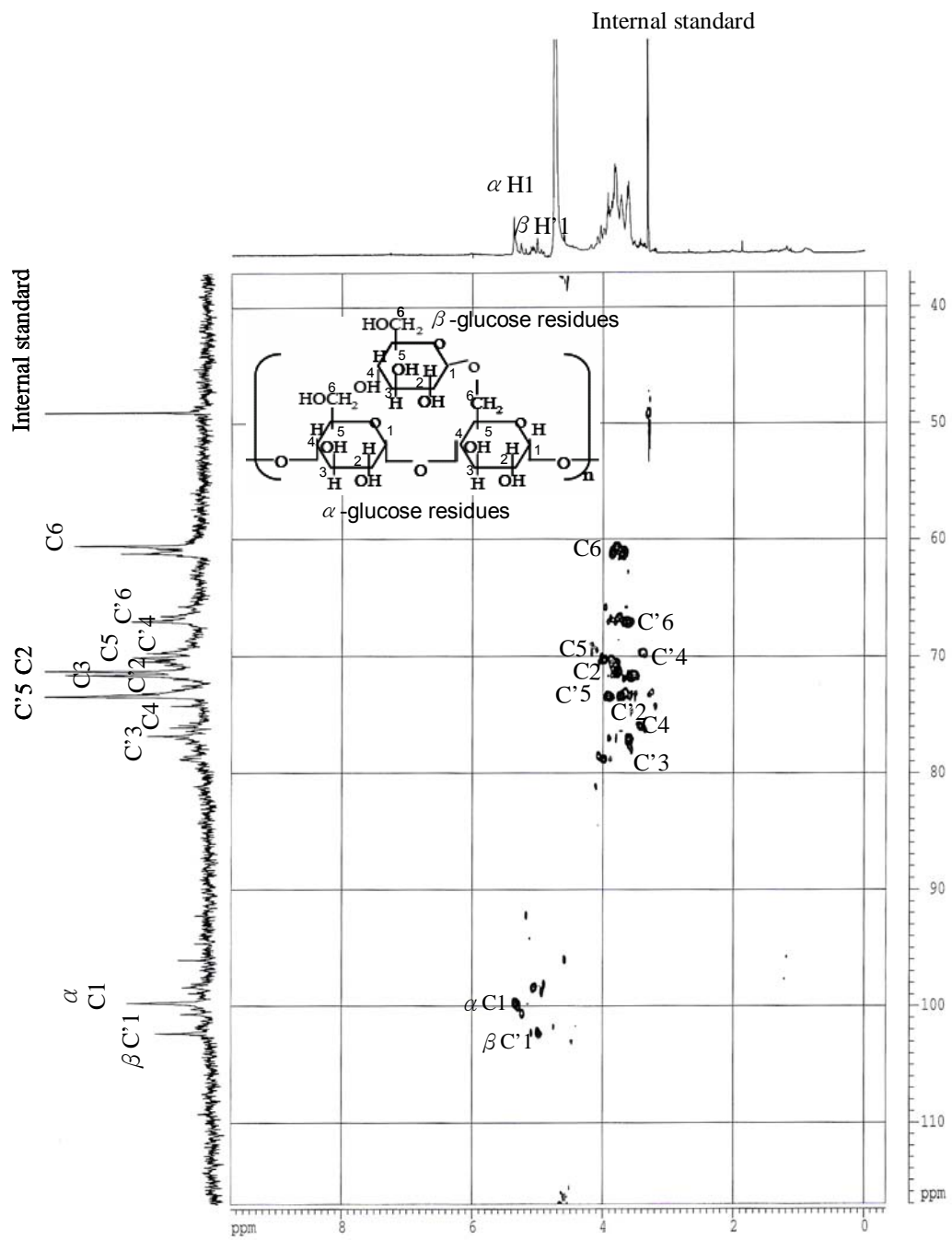


Fig. 14 The 2D (HSQC) NMR spectrum of the L15 BWE in D₂O.

PART II

Pressurized Water Extraction of Polysaccharides as Secondary Metabolites from *Lentinula edodes*

Abstract

The suitability of pressurized water extraction (PWE) of crude polysaccharides as secondary metabolites from *L. edodes* was investigated. A series of experiments were carried out to examine the effects of extraction times and pressures. The results indicated that the maximum recovery of polysaccharides was about 90% of the crude polysaccharides from mycelia pellets when the pressure was at 10.1 MPa for 70 min (28 °C). This was a drastic improvement over that of boiling water extraction (BWE) at 0.1 MPa for 40 min, which gave only 27.9% recovery. A nitroblue tetrazolium (NBT) reduction assay was used to examine the macrophage stimulating activities (MSA), and it was found that the PWE polysaccharides retained the MSA. The morphology of the macrophage cells treated by PWE polysaccharides was also examined and found to be similar to that of the positive control lipopolysaccharides treated. Finally, gel chromatographic and NMR experiments revealed that both PWE and BWE polysaccharides showed the presence of four similar molecular mass components and the α -(1→4)-D-Glcp and β -(1→6)-D-Glcp linkage residues. The improved PWE

efficiency is probably due to the possibility that under high pressure, the solid polysaccharide's hydrogen bonding is partially destroyed to increase structure elasticity and water solubility.

Results and discussion

Optimization of *L. edodes* Culture Growth and PWE of Polysaccharides.

Fig. 1 shows the time course of mycelia growth curve expressed as plots of dry weights versus time. The results indicated that the mycelia dry weight reached 461.2 mg/50 mL at the 12th day and that the growth rate of mycelia could be roughly divided into three phases. The first one appeared from the 3rd to the 7th day and had a slow growth rate. The second one appeared from the 7th to the 12th day with the best mycelia growth rate. After the 14th day, the mycelia growth moved to a stationary phase. The MSA measured by using the NBT reduction assay on the collected culture broth filtrates (CBF) from the mycelia culture at various days are also plotted in Fig. 1. Although the MSA was slightly greater for the 17th day polysaccharides, because of the considerations of (1) the quantity of polysaccharides, (2) polysaccharide bioactivity, and (3) time, the operational time to harvest the mycelia growth and crude polysaccharides production were set at the 14th day.

BWE (40 min at 0.1 MPa, as control experiments) and PWE (between 10 and 80

min at 25.3 MPa) of the crude polysaccharides as secondary metabolites from *L. edode* culture broth filtrate were performed to obtain the optimum extraction time; 25.3 MPa was the highest pressure the instrument could achieve. Under this pressure the optimum extraction time was obtained, and at this optimum extraction time, the pressure was then reduced to find the optimum extraction efficiency (48, 49). It was found that BWE recovered only 27.9% of the crude polysaccharides, which was much less than those extracted with PWE at all time conditions (Table 1). When the PWE time was increased from 10 to 70 min, the polysaccharide recovery increased from 80.6 to 88.6%. The greatest recovery of polysaccharides was 88.6% at a pressure of 25.3 MPa for 70 min. As shown in Table 1, increasing the extraction time to 80 min did not increase polysaccharide recovery. Thus, extraction time was set at 70 min for subsequent experiments. Table 1 also shows the polysaccharide extraction data at various pressures at 70 min. The recoveries of polysaccharides slightly increased from 85.1% at an extraction pressure of 2.5 MPa to 90.0-90.3% at 10.1-15.2 MPa and then diminished to 88.6% at 25.3 MPa. A maximum recovery (~90%) at 10.1 MPa was obtained. Thus, an optimum condition for PWE was therefore set at 10.1 MPa and 70 min. At 2.5-25.3 MPa, the yield (i.e., solubility) of polysaccharides was about 29.8-31.6 mg/10 mL (calculated from the initial 35 mg/10 mL of crude polysaccharides). The extraction reproducibility was expressed by RSD (%). It was

found that for BWE, the RSD was 21.5%, which was much greater (and hence less reproducible) than those of PWE, which were normally <2.7%. This was due, probably in part, to the lack of precise control of pressure and temperature under the traditional extraction processes.

Generally, the extracted amount of polysaccharides depends on temperature, pressure, pH of the solvent, ionic strength, and fineness of solid particle grinding. The polysaccharide Lentinan was first isolated and studied by Chihara (81). The extractions were performed using 80-100 °C boiling water for 8-16 h. Mizuno et al. later modified the extraction method, and the extractions of high molecular mass polysaccharides were performed with three successive steps, that is, with water (100 °C, 3 h), 2% ammonium oxalate (100 °C, 6 h), and 5% sodium hydroxide (80 °C, 6 h) (1, 44). A strong base such as NaOH alone in the extraction medium resulted in a higher extraction yield than pure water at all temperatures tested (45, 99, 100). This modified method was more efficient and relatively less costly when compared to the original Chihara method (46, 81, 101). However, the use of boiling water was not convenient, and dilute alkaline solution may alter the structures and bioactivities of the polysaccharides (45). Note that the -OH groups in the anhydrous glucose units are ionized at high pH (i.e., pH 12.5), which might increase the polysaccharides' water solubility and structure elasticity in addition to the reduction of polymer chain

agglomeration and molecular degradation (102, 103).

MSA and Macrophage Morphology.

It has been reported that polysaccharides possess an immunomodulating effect by the activation of a variety of macrophage functions, for example, superoxide anion production and cytokine production (104, 105). The MSA of the PWE polysaccharides were examined by using the NBT assay, and the results are shown in Fig. 2. The NBT reduction of macrophage activities at 5.1, 7.6, 10.1, 15.2, and 25.3 MPa extractions were 148, 166, 172, 192, and 207%, respectively. These data were close to that of BWE (151%), indicating that both the PWE and BWE methods were able to retain the bioactivities of the polysaccharides. It is noted that CBF has higher macrophage activities, probably due to the presence of other metabolites in addition to polysaccharides.

When macrophage cells were cultivated for 24 h with LPS, BWE, or PWE polysaccharides, dramatic morphological changes were observed (Fig. 3). The LPS is a cell wall component of a Gram-negative bacteria and can activate monocytes and macrophages (106, 107). Figure 4b shows that the macrophage cells appeared to have distinct dendritic-like morphology after 24 h of culturing in the presence of the LPS positive control as compared with those of unstimulated macrophage cells (Fig.

3a). Macrophage cells cultured with both BWE and PWE polysaccharides show morphology similar to those of the LPS treated (Fig. 3c, d). The macrophage cells in Figure 4b-d were characterized by a dramatic increase in size, larger nuclei, prominent nucleoli, extended processes, and cytoplasm with increased granularity as compared with untreated macrophage cells.

Molecular Mass Determinations.

Gel permeation chromatographic studies show that the BWE and PWE polysaccharides have four similar molecular mass fractions (designated A, B, C, and D, Table 2, Fig. 4). The polysaccharides show a broad molecular mass range from 10 to >2750 kDa. Careful examinations revealed that these molecular mass fractions A (>2750 kDa), B (~2700 kDa), C (~534 kDa) and D (~11.7 kDa) were present in all extracts with minor variations in fractions ratios (area %).

The PWE was utilized to improve the extraction yields of polysaccharides from solid or semisolid samples. High pressure could modify considerably the physical properties of the extraction solvents, with the effect of increasing selectivity in the extraction (52). The PWE could minimize strong hydrogen bonding of solid crude polysaccharides and increase structure elasticity to increase water solubility without changing most polysaccharide structures and therefore, similar molecular mass

distributions and bioactivities were found after the extraction (25-250 atm, 10-80 min).


NMR studies.

The proton and ^{13}C NMR spectra of the PWE polysaccharides (100 atm, 70 min.) are shown in Fig. 5. The two groups of anomeric proton signals centered at 5.36 and 5.01 ppm (Fig. 5A) were assigned to the α -(1 \rightarrow 4)-D-Glcp and β -(1 \rightarrow 6)-D-Glcp protons, respectively (71, 77). These assignments of α -(1 \rightarrow 4)-D-Glcp and β -(1 \rightarrow 6)-D-Glcp protons were similar to those of BWE at 5.33 and 4.97 ppm. The assignments of the C1 and C'1 chemical shifts of the glucose residues were reported in the range of 98~104 ppm with the α form being at downfield and β form at upfield (77, 108, 109). The two carbon peaks at 99.67 and 102.27 ppm were assigned to α -(1 \rightarrow 4)-D-Glcp and β -(1 \rightarrow 6)-D-Glcp carbons, respectively (Fig. 5B, Table 3). It should be noted that although the chemical shifts of the two C1 peaks of the backbone glucose units may theoretically be different, in our hands and others reported, they were found to be similar.

The anomeric carbon peaks C1 and C'1 of the BWE polysaccharides were similar to those of PWE and the corresponding chemical shifts were at 99.68 and 102.43 ppm. The peaks at 71.56, 71.31, 76.06, 70.07, and 60.87 ppm were assigned, respectively, to C2, C3, C4, C5, and C6 of the α -(1 \rightarrow 4)-D-Glcp carbons, due to their

relatively higher peak intensities. The other signals at 73.20, 76.80, 69.72, 73.42 and 66.97 ppm were assigned, respectively, to C'2, C'3, C'4, C'5, and C'6 of the β -(1 \rightarrow 6)-D-Glcp carbons. The tentative ^{13}C NMR peak assignments for the mannopyranosyl carbons are: C"1 (96.02 ppm), C"2 (69.65 ppm), C"3 (70.35 ppm), C"4 (78.5 ppm), C"5 (74.25 ppm) and C"6 (61.2 ppm) based on those assignments of related studies (37, 110). The assignment of the peak at 92.0 ppm was not certain but could be attributed by a monosaccharide carbon residue (111).

Conclusion



In conclusion, although some previous studies indicated that increased extraction yields of polysaccharides were obtained by adjusting the pH or other extraction conditions, few reported if the structures and bioactivities were the same or similar before and after the improvement of extraction yield. The data presented in this paper indicated that the yield of the polysaccharides extracted by the PWE method could be increased up to 90% of the total crude polysaccharides from CBF, which was about three times of that by the BWE method. Moreover, the extracted polysaccharides by both the PWE and BWE methods showed similar macrophage stimulating activities, macrophage morphologies, molecular mass distributions and NMR spectral properties. The PWE method for polysaccharides is easy to practice

and is recommended for future applications in agricultural and food research and industrial product developments.



Tables

Part II

Table 1. Recoveries of crude polysaccharides by pressurized water extraction (PWE) in different time periods and pressures. Boiling water extractions (BWE) were performed at 0.1 MPa for 40 min. Data were the average of triplicate experiments.

Pressure (MPa)	Time (min)	Recovery \pm R.S.D., %
0.1 (BWE)	40	27.9 \pm 21.0
25.3	10	80.6 \pm 1.5
25.3	20	81.4 \pm 1.3
25.3	30	82.0 \pm 0.9
25.3	40	85.8 \pm 1.4
25.3	50	85.8 \pm 1.0
25.3	60	87.5 \pm 0.9
25.3	70	88.6 \pm 1.3
25.3	80	88.6 \pm 0.5
2.5	70	85.1 \pm 2.7
5.1	70	85.7 \pm 1.0
7.6	70	85.8 \pm 0.4
10.1	70	90.0 \pm 1.5
15.2	70	90.3 \pm 1.2

Table 2. Selected gel-chromatographic retention data of the BWE and PWE polysaccharides. A (>2750 kDa), B (~2700 kDa), C (~534 kDa) and D (~11.7 kDa).

Sample	Fractions	Retention time (min)	% Area
BWE (0.1 MPa)	A	5.7	12.0
	B	8.2	23.1
	C	9.2	18.8
	D	10.1	46.2
PWE (2.5 MPa)	A	5.6	12.2
	B	8.3	25.7
	C	9.2	15.8
	D	10.6	46.3
PWE (10.1 MPa)	A	5.7	12.4
	B	8.3	22.3
	C	9.2	16.1
	D	10.6	49.3
PWE (25.3 MPa)	A	5.7	12.6
	B	8.4	25.3
	C	9.2	16.8
	D	10.6	45.3

Table 3. ^{13}C NMR chemical shifts of BWE and PWE polysaccharides in D_2O .

α -(1→4)-D-Glcp	C1	C2	C3	C4	C5	C6
BWE	99.68	71.55	71.28	76.06	70.09	60.86
PWE	99.67	71.56	71.31	76.06	70.07	60.87

β -(1→6)-D-Glcp	C'1	C'2	C'3	C'4	C'5	C'6
BWE	102.43	73.16	76.79	69.75	73.45	66.96
PWE	102.27	73.2	76.8	69.72	73.42	66.97



Figure Captions

Part II

Fig. 1: Plots of isolated mycelia dry weights (\blacktriangle , mg/50ml) and the macrophage stimulating activities (\bullet , % NBT reduction) of the culture broth filtrates (CBF) vs. time during the mycelia growth of *L. edodes*.

Fig. 2: Macrophage stimulatory activities of PWE polysaccharides extracted at different pressures.

Fig. 3: DIC (differential interference contrast) images of macrophage morphological changes induced by (a) PBS, (b) 10 μ g/ml LPS, (c) BWE and (d) PWE polysaccharides.

Fig. 4: Gel permeation chromatograms of PWE and BWE polysaccharides. Molecular mass fractions: A (>2750 kDa), B (~2700 kDa), C (~534 kDa) and D (~11.7 kDa).

Fig. 5: NMR spectra of PWE polysaccharides (100 atm, 70 min) in D₂O: (a) ¹H NMR and (b) ¹³C NMR.

Part II

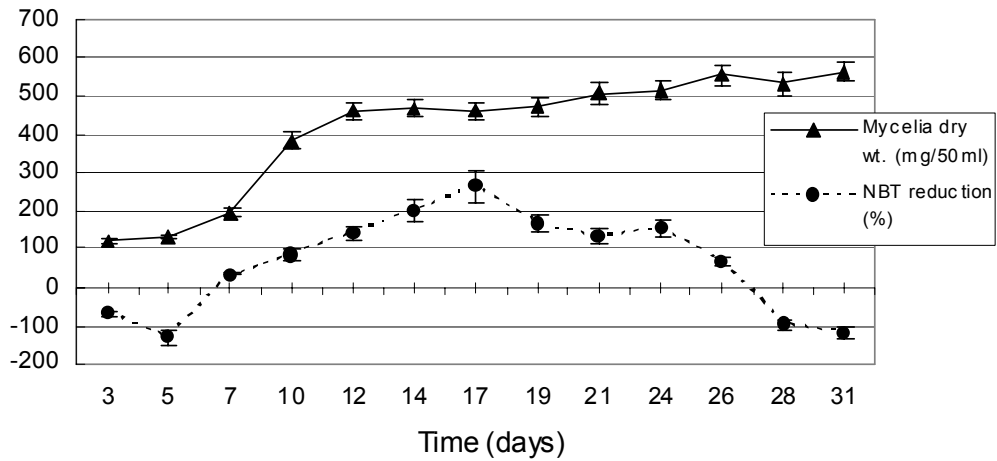


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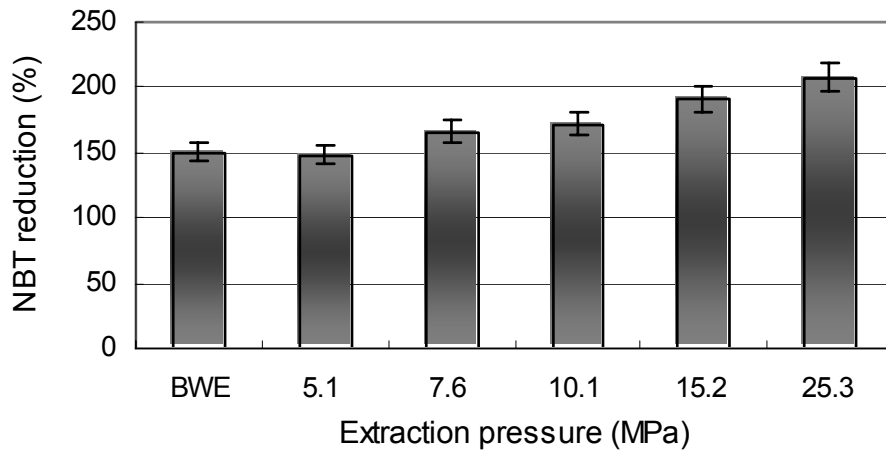


Fig. 2 Macrophage stimulatory activities of PWE polysaccharides extracted at different pressures.

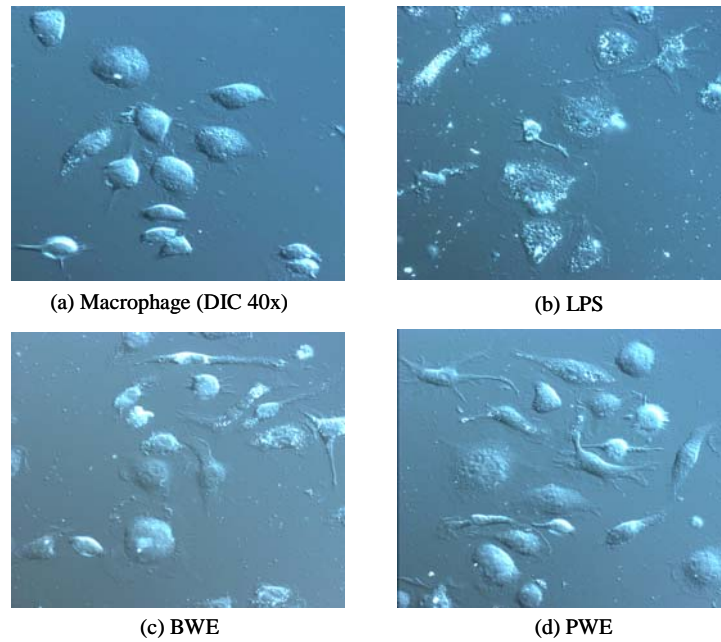


Fig. 3 DIC (differential interference contrast) images of macrophage morphological

changes induced by (a) PBS, (b) 10 $\mu\text{g/ml}$ LPS, (c) BWE and (d) PWE

polysaccharides.

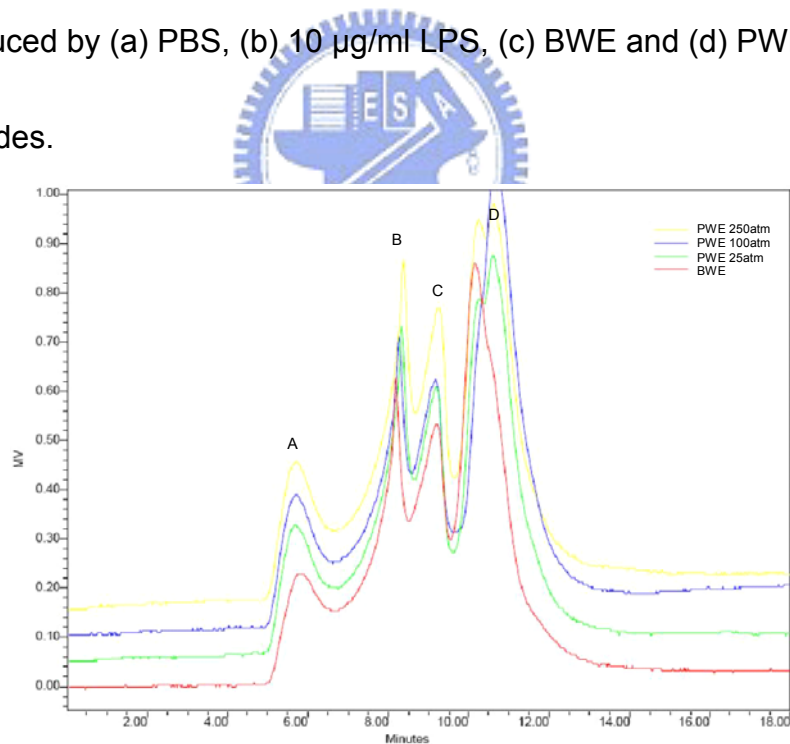
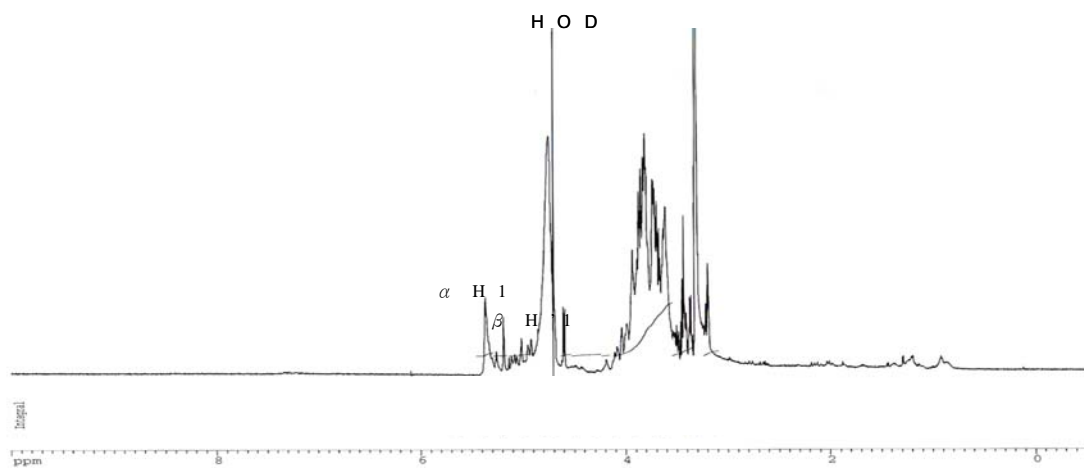
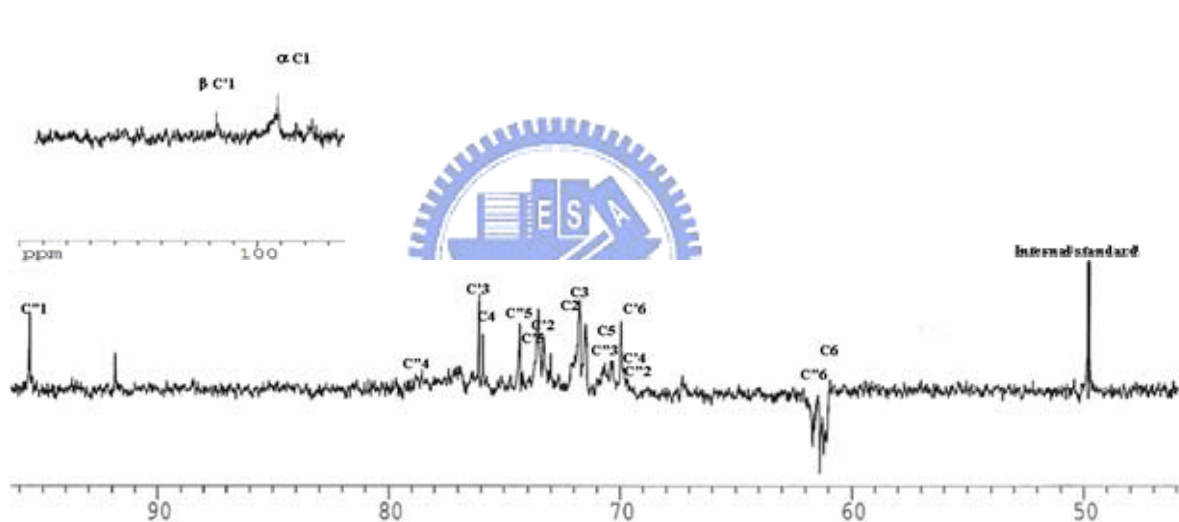


Fig. 4 Gel permeation chromatograms of PWE and BWE polysaccharides.

Molecular mass fractions: A (>2750 kDa), B (~2700 kDa), C (~534 kDa) and D (~11.7 kDa).



(A)



(B)

Fig. 5 NMR spectra of PWE polysaccharides (100 atm, 70 min) in D₂O: (a) ¹H NMR and (b) ¹³C NMR.

PART III

Use of Statistical Methods to Find the Polysaccharide Structural Characteristics and the Relationships between Monosaccharide Composition Ratio and Macrophage Stimulatory Activity of Regionally Different Strains of *Lentinula edodes*

Abstract

Multiple linear regression analysis was used to deduce the correlation between the monosaccharide composition ratios of ten regionally different strains of *L. edodes* and their *in vitro* macrophage stimulatory activities. Arabinose, xylose, mannose, fucose and galactose were identified as the monosaccharides that could be related to macrophage stimulatory activities. Additional principal component analysis and factor analysis methods were used to treat the same monosaccharide composition ratio data and the compositions of arabinose, xylose, mannose and galactose were found to be important. Interestingly, glucose, although presented in large compositions in all strains presumably forms the backbone of the polysaccharide structures, is not selected as the determinant factor for either structural characteristics or that of the *in vitro* macrophage stimulatory activities.

Results and discussion

Multiple linear regression analysis.

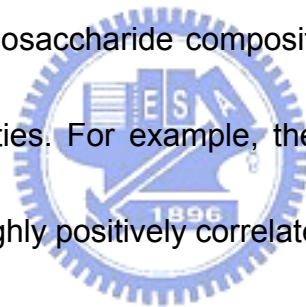
The method adopted here allowed us to detect major monosaccharide composition differences between the ten strains and to predict characteristic components of each polysaccharide immuno-competence. Using multiple regression backward analysis, the P-values for testing linear detected relationship were (a) arabinose < 0.001, (b) xylose < 0.001, (c) mannose < 0.001, (d) galactose = 0.007, (e) Fucose < 0.001. For other monosaccharides, the P-values were > 0.1. A P-value < 0.05 was considered to be statistically significant. The coefficient of determination (R^2) which measures the model fit was 0.949. To predict relationship between monosaccharide ratio and MSA, equation 7 was obtained:

$$Y (\% \text{ macrophage activity}) = 0.138 + 1.668X_{Ara} - 1.140X_{Xyl} - 0.051X_{Man} - 0.0995X_{Gal} - 0.743X_{Fuc} \quad (7)$$

where 'X' defines the concentration of monosaccharide measured in the extract. The positive coefficient for X_{Ara} indicates that the macrophage stimulatory activity increases with increasing X_{Ara} . The negative coefficients for X_{Xyl} , X_{Man} , X_{Gal} , and X_{Fuc} indicate that the macrophage stimulatory activity decreases with the corresponding increasing X_{Xyl} , X_{Man} , X_{Gal} , and X_{Fuc} .

The scatter plot matrix showed that arabinose and xylose were rather highly

positively correlated (Pearson correlation = 0.971) and meaning that in going from one polysaccharide to the other, a higher value of X_{Ara} was found normally with a higher value of X_{Xyl} . These kind of positive correlations were also found among (a) arabinose and xylose, mannose, galactose, (b) xylose and mannose, galactose, (c) mannose and galactose, (d) glucose and rhamnose. On the other hand, negative correlations were found among (a) glucose and arabinose, xylose, mannose, galactose, (b) rhamnose and arabinose, xylose, mannose, galactose (Fig. 1). In these cases, a higher value of, e.g. X_{glc} , was accompanied by lower values of X_{Ara} , X_{Xyl} , X_{Man} , and X_{Gal} . Note that positive monosaccharide composition correlations do not necessarily lead to positive MSA activities. For example, the monosaccharide compositions of arabinose and xylose are highly positively correlated for these ten strains of *L. edodes*; however, arabinose has a positive MAS coefficient but xylose has a negative one.



The accuracy of the multiple linear regression analysis results was further tested by residual analysis (78). The residual is defined as follows:

$$\text{Residual} = \text{observed value} - \text{predicted value} \quad (8)$$

The normal P-P plot of regression residuals using SPSS was obtained in order to assess whether the normality assumption was violated. The plot that supports the normality assumption was constructed for the cumulative proportions of residuals against the cumulative proportions of the normal distribution (Fig. 2A). The normal

probability plot showed a light tail distribution plot indicating normality is approximately valid. To check if the assumption of constant variance is not violated, the residuals were plotted against the predicted values. The change in the spread or dispersion of the plotted points could be used to detect whether the constant variance was satisfied or not. As seen in Fig. 2B, the points were dispersed in a non-systematic way, confirming the reliability of predicted model.

Principal component analysis and Factor analysis.

The arabinose, xylose, mannose and galactose (eigenvalue = 4.71) were the dominating features in the first principal component (Prin1) that represented 67 % of the monosaccharide composition property. Monosaccharides such as rhamnose and fucose (eigenvalue = 1.39) were features in the second principal component (Prin2) that represented 20 % of the monosaccharide composition property. The correlation matrix, eigenvectors and eigenvalue data were shown in Table 2A and 2B. The PCA two-dimensional plot (i.e. Prin1 vs. Prin2, Fig. 3) revealed that L1, L4, L11, L24 and L25 were located further from the rest, which happened to be the group of relatively poorer MSA (macrophage stimulating activity). Those with better MSA, i.e. L6, L10, L15, L21 and L23, are located closer to the line of Prin1 = 0. Thus, PCA analysis, although designed to find only structural characteristics, was able to classify these polysaccharides roughly into a more active MSA group and a less active MSA one

indirectly.

A third test using factor analysis (FA) indicated that the composition of arabinose, xylose, mannose and galactose were important structural characteristics among these polysaccharides (Table 3 and Fig. 4). These four monosaccharides were identical to those found for Prin1 in PCA, which further corroborated the generally believed consistent results from both PCA and FA methods.

Conclusions

Multiple linear regression analysis method was beneficial to deduce the correlation between monosaccharide composition and macrophage stimulating activity data of ten different strains of *L. edodes*. The principal component analysis and factor analysis methods were useful to find polysaccharide structural characteristics. Our present data and previous reports all pointed out that arabinose, xylose, mannose and galactose were related to biological functions and were unique polysaccharide structural characteristics of *L. edodes* (1, 55, 56). Specifically, the present study showed that the three different statistical methods, although applied for different purposes, generated consistent polysaccharide structural characteristics and both direct and indirect relationships between monosaccharide composition ratios and macrophage stimulating activities. Because it is not always possible to generate large quantity of experimental data, it is recommended that more than one statistical

method are used for data treatment for more objective results, as shown in this study.

Although the enhanced macrophage activity was found to correlate to the complex polysaccharide structural characteristics, the actual molecular mechanisms involving cell signal transduction and more precise structure-function relationships remained to be explored. Interestingly, glucose, although presented in large compositions in all strains presumably forms the backbone of the polysaccharide structures, is not selected as the determinant factor for either structural characteristics or that of the *in vitro* macrophage stimulatory activities.

The elucidation of polysaccharide structures and biological activities normally involves complex instrumental techniques and various biological assays. For potential applications of this present study, it is possible to simplify the above-mentioned complexity by just determining the monosaccharide composition ratios and predicting the macrophage stimulatory activity using the established structure-function relationships.

Tables

Part III

Table 1. Monosaccharide composition and MSA of culture broth filtrate of different strains of *Lentinula edodes*. (A) Selected raw data. (B) Operation data.

(A)

<i>L. edodes</i>	Monosaccharide compositions (molar ratio)							Optical density
	Arabinose	Xylose	Mannose	Galactose	Glucose	Rhamnose	Fucose	NBT reduction
L1	0.46	0.37	2.45	0.11	4.2	0	0	0.37
L4	0.79	0.75	3.46	0.36	1.62	0.02	0.04	0.35
L6	0.49	0.35	1.89	0.15	3.03	0.01	0.01	0.43
L10	0.57	0.41	1.98	0.26	4.13	0	0	0.49
L11	0.59	0.46	3.19	0.38	4.14	0.01	0.02	0.4
L15	0.59	0.42	2.17	0.15	7.47	0.03	0.01	0.52
L21	0.5	0.35	1.99	0.22	2.81	0.03	0.03	0.44
L23	0.54	0.39	1.88	0.22	3.96	0.02	0.01	0.5
L24	0.27	0.14	0.89	0	10.9	0.06	0.02	0.38
L25	0.42	0.29	2	0.12	3.59	0.03	0.02	0.36

(B)

<i>Lentinula edodes</i>	L1	L4	L6	L10	L11	L15	L21	L23	L24	L25
Arabinose %	6.03	11.19	8.25	7.77	6.76	5.41	8.42	7.73	2.22	6.45
Xylose %	4.82	10.65	5.89	5.58	5.21	3.83	5.88	5.49	1.13	4.55
Mannose %	32.28	49.22	31.83	26.89	36.24	20.02	33.5	26.72	7.23	30.91
Galactose %	1.44	5.09	2.59	3.56	4.3	1.42	3.73	3.19	0.03	1.87
Glucose %	55.44	23.1	50.95	56.2	47.12	68.93	47.36	56.36	88.82	55.58
Rhamnose %	0	0.22	0.24	0	0.14	0.28	0.56	0.32	0.45	0.4
Fucose %	0	0.52	0.24	0	0.24	0.11	0.56	0.19	0.12	0.24
Exopolysaccharide Content (mg/ml)	0.58	0.61	0.53	0.31	0.48	0.44	0.2	0.15	0.59	0.59
MSA (% NBT reduction)	166	150	317	449	230	489	330	464	200	153

Table 2. Correlation data of the correlation matrix (A) and eigenvectors (B) for the seven variables.

	Arabinose	Xylose	Mannose	Galactose	Glucose	Rhamnose	Fucose
(A) Correlation matrix							
Arabinose	1	0.971	0.842	0.864	-0.628	-0.516	0.4
Xylose	0.971	1	0.886	0.815	-0.645	-0.507	0.449
Mannose	0.842	0.886	1	0.825	-0.649	-0.589	0.386
Galactose	0.864	0.815	0.825	1	-0.656	-0.51	0.439
Glucose	-0.628	-0.645	-0.649	-0.656	1	0.662	-0.319
Rhamnose	-0.516	-0.507	-0.589	-0.51	0.662	1	0.373
Fucose	0.4	0.449	0.386	0.439	-0.319	0.373	1
	Prin1	Prin2	Prin3	Prin4	Prin5	Prin6	Prin7
(B) Eigenvectors							
Arabinose	0.434	0.059	0.332	-0.185	-0.483	0.391	-0.527
Xylose	0.437	0.085	0.278	-0.462	-0.172	-0.39	0.574
Mannose	0.428	0.002	0.187	-0.069	0.814	0.339	0.001
Galactose	0.421	0.071	0.105	0.854	-0.153	-0.05	0.225
Glucose	-0.368	0.135	0.813	0.132	0.152	-0.323	-0.201
Rhamnose	-0.292	0.647	0.104	-0.016	-0.104	0.554	0.409
Fucose	0.197	0.74	-0.307	-0.024	0.134	-0.408	-0.367
Eigenvalues	4.706	1.394	0.494	0.202	0.176	0.02	0.009



Table 3. Factor pattern data for Factor 1 and Factor 2.

Factors/monosaccharides	Factor 1	Factor 2
Xylose	0.947	0.1
Arabinose	0.942	0.069
Mannose	0.929	0.002
Galactose	0.914	0.084
Glucose	-0.799	0.159
Fucose	0.427	0.874
Rhamnose	-0.634	0.764

Figure Captions

Part III

Fig. 1: 2D scatter plot of the relationship between all monosaccharide compositions and macrophage activity (NBT reduction).

Fig. 2: (A) Normal P-P plot of regression residuals (dependent variable: NBT reduction). (B) Plot of residuals versus predicted for the NBT reduction data.

Fig. 3: PCA for composition data: relationship among different strains. Plot of Prin1 and Prin2.

Fig. 4: Plot of factor pattern for Factor1 and Factor2.



Part III

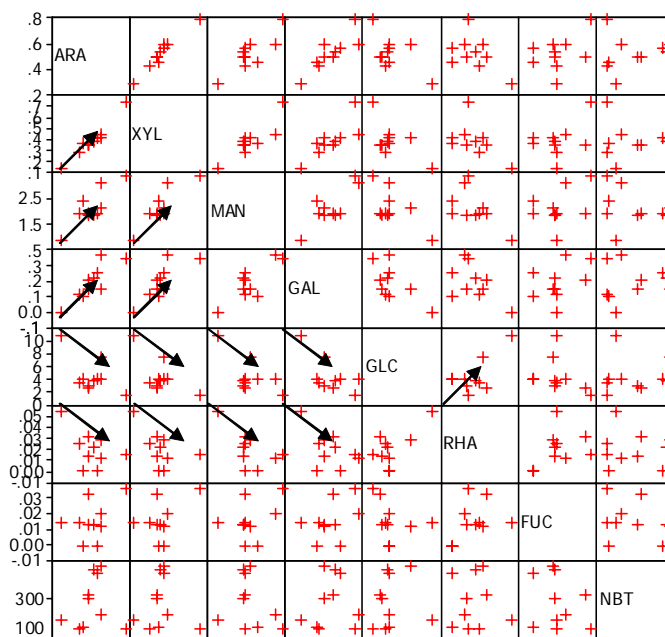


Fig. 1 2D scatter plot of the relationship between all monosaccharide compositions and macrophage activity (NBT reduction).

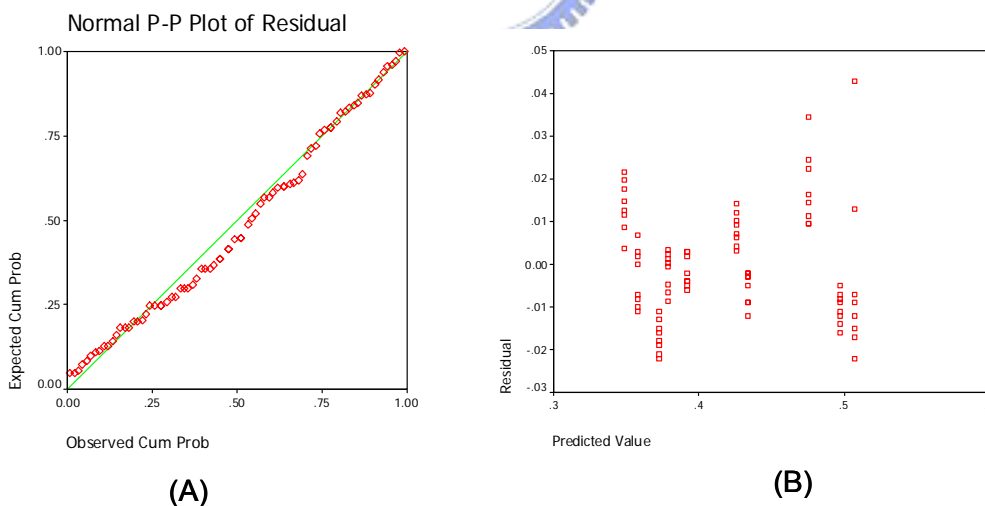


Fig. 2 (A) Normal P-P plot of regression residuals (dependent variable: NBT reduction). (B) Plot of residuals versus predicted for the NBT reduction data.

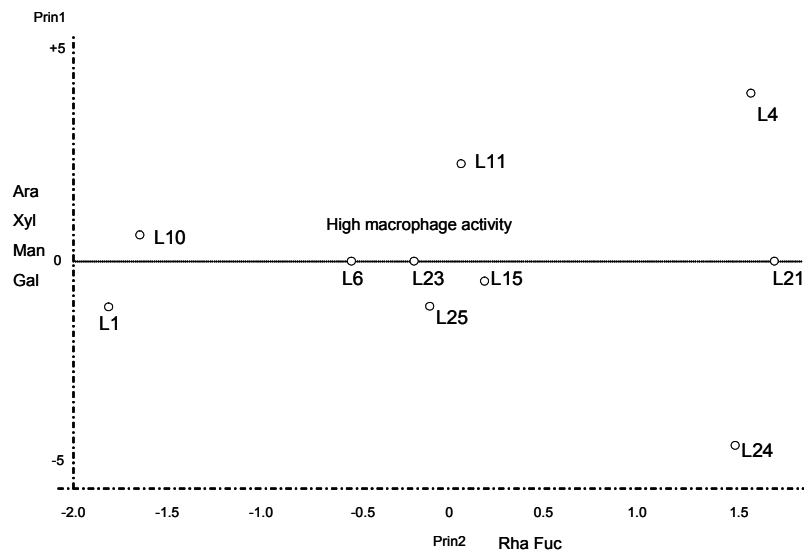


Fig. 3 PCA for composition data: relationship among different strains. Plot of Prin1 and Prin2.

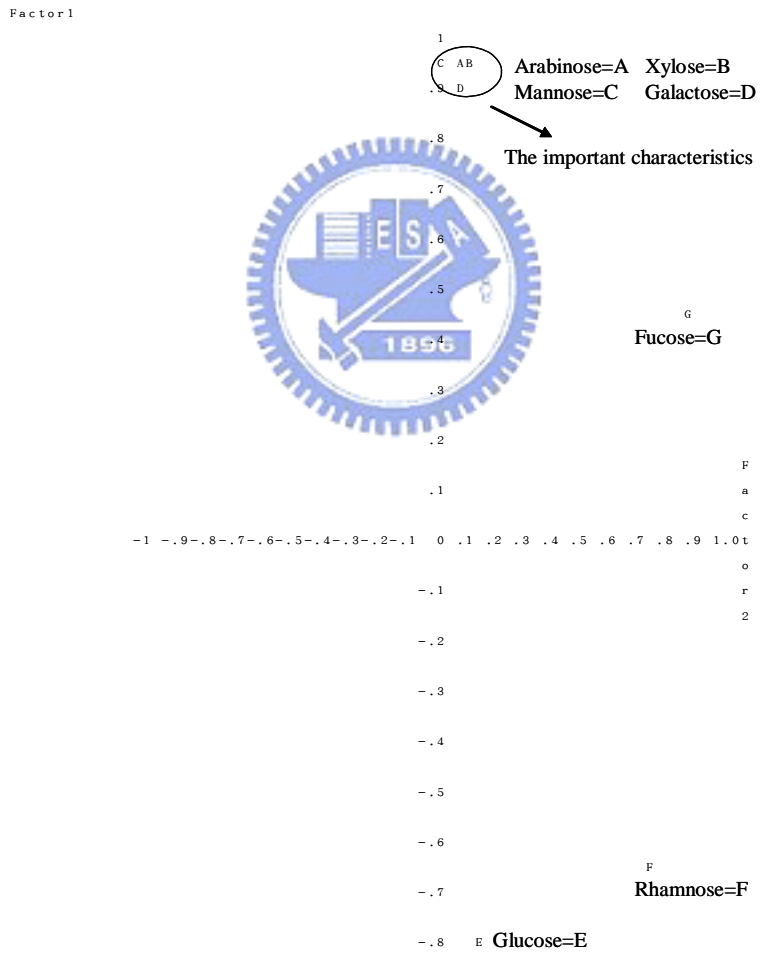


Fig. 4 Plot of factor pattern for Factor1 and Factor2.

PARTIV

Glycosyl Linkage Characteristics and Classifications of Exo-polysaccharides of Some Regionally Different Strains of *Lentinula edodes* by Amplified Fragment Length Polymorphism Assay and Cluster Analysis

Abstract

We report here the first combined amplified fragment length polymorphism (AFLP) assay and cluster analysis of the exo-polysaccharide glycosyl linkage data of ten regionally different strains of *L. edodes* to compare their genetic and structural similarities and differences. In addition, the monosaccharide compositions, molecular mass, glycosyl structural linkages were investigated for the exo-polysaccharides extracted from these different phylogenetic groups of regionally different *L. edodes*. All exo-polysaccharides had similar molecular mass distribution between 1×10^4 and 3×10^6 Da and the monosaccharide composition analysis revealed the presence of heterogeneous materials containing glucose, mannose, xylose, galactose, fucose, rhamnose and arabinose in different ratios. Among these monosaccharides, the glucose contents are the highest for all but one strain, indicating that glucose probably is the building block of the backbones of these exo-polysaccharides. The AFLP assay

data helped to classify the ten *L. edodes* strains into three distinct genetic groups.

Gas chromatographic and mass spectrometric (GC-MS) data revealed five different glycosyl linkage types for these exo-polysaccharides. Most of the exo-polysaccharide backbone structures contain (1→4)-linked-D-glucopyranosyl and (1→6)-linked-D-glucopyranosyl moieties. Arabinose 1→4 linkages and mannose 1→2 linkages also exist in all strains. The only differences among these linkages are their monosaccharide compositions leading to different degree of backbone and branch formations. Cluster analyses of the GC-MS data of the exo-polysaccharides of the ten strains resulted in ten dendrograms. However, four of the ten dendrograms were identical and were obtained using the Average, Ward and Weighted linkage type method of Manhattan distance and using the Ward method of Euclidean distance. The results of cluster analyses were not very much different from that of the AFLP assay and allowed the comparison of genetic and structural similarities and differences.

Results and discussion

***L. edodes* culture.**

Recently, the submerged cultivation of mushroom has received much attention in Asian regions as a promising alternative for efficient production of its valuable metabolites, especially polysaccharides and ganoderic acids (38, 88). Traditionally, it

usually takes several months to cultivate the fruiting body of the mushroom, and it is also difficult to control product quality during soil cultivation. There is a great need to supply the market with a large amount of lower cost and high-quality mushroom products. Therefore, submerged cultivation of mushroom could eventually supplement the need and prove useful over fruiting body cultivation.

The optimum environmental conditions for our current submerged culture growth and exo-polysaccharide production of mushrooms in liquid cultures are dependent on strains. Initially, the growth conditions of the culture were optimized that showed a stationary phase after 14 days. The biomass of the culture broth filtrate extracts of all strains were weighed to be in the range of 0.35 to 0.40 g/50 ml and exo-polysaccharide contents between 0.15 and 0.59 mg/ml (Table 1). These values would be useful as references for optimization and pilot plant production studies in the future (65).

Strain typing/phylogenetic mapping.

Fig. 1 shows the AFLP fingerprints of genomic DNAs of different *L. edodes* strains. A dendrogram of the similarity index based on the bands obtained for selected two primer sets was plotted to distinguish the closely related strains as shown in Fig. 2. The ten isolates of *L. edodes* were grouped into three distinct clusters by AFLP: (1)

L24 and L25 isolates from China; (2) L1 and L4 isolates from Taiwan; (3) L6, L10, L11, L15, L21 and L23 isolates from Japan. A very close genetic homogeneity among cultivated strains of Japanese mushrooms, L6, L21, L10, L11, L15, and L23 was observed. Similarly, for the Taiwanese (L1 and L4) and Chinese (L24 and L25) mushrooms, the obtained AFLP fingerprints point out close resemblance to the genetic homogeneity. However, L24 and L25 mushrooms from China were quite different from the Japanese and Taiwanese mushrooms. The mushrooms, L1 and L4 were cultivated as hybrid strains comprising of Japanese strains SL-19 and 271.

The dendrogram obtained using AFLP (fingerprinting) analysis of *L. edodes* provides new insight into the population structure of this mushroom species and proves useful for phylogenetic type studies. The results appear promising and are well supported by results obtained using random amplified polymorphic DNA (RAPD) assay (fingerprinting) for the same species (89).

However, the time and cost efficiency, reproducibility and resolution of AFLP are superior or at least equal to those of other markers, e.g. RAPD, allozymes, restriction fragment length polymorphism (RFLP) and microsatellites (112). Therefore, application of AFLP fingerprint assay for phylogenetic studies of mushrooms can now be included among other reported species in population studies.

Molecular mass and monosaccharide composition.

The results of molecular mass analysis indicated that all extracted polysaccharide materials fell into four molecular mass fractions: A (>2750 kDa), B (~2700 kDa), C (~534 kDa) and D (11.7 kDa) (Table 2 and Part I Fig. 10). All exo-polysaccharides had similar molecular mass distribution between 1×10^4 and 3×10^6 Da.

The results showed that great differences existed in the distribution ratio of the high molecular mass fractions A. The contents of each fraction in decreasing order are listed below: A (> 2750 kDa): L15 ~ L10 > L25 > L23 ~ L4 > L6 ~ L21 > L24 > L11 > L1; B (~2700 kDa): L10 > L25 > L24 > L15 ~ L4; C (~534 kDa): L11 > L1 > L23; and D (~11.7 kDa): L24 > L4 > L25 > L10 > L6 > L1 > L15 > L21 ~ L23 > L11. The contents of exo-polysaccharide materials are complex in nature and poses difficulties for better understanding of their importance and structure-function relationships (*vide infra*).

The monosaccharide composition data are listed in Table 3. These data revealed great differences in the distribution of glucose, mannose, xylose, galactose, fucose, rhamnose and arabinose of the crude polysaccharide of all mushrooms, strongly indicating that these materials are heterogeneous (1). Except for L24, the glucose contents of all other strains are the greatest. This presumably indicates that the polysaccharides backbone and branched side chains are formed by mainly

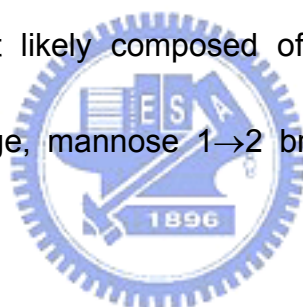
glucose-1→4 and 1→6-linkages, respectively. For L4 polysaccharide, the mannose composition is the greatest among all found monosaccharides, probably indicating that the backbone and/or branch contain more mannose 1→2-linkages (*vide infra*).

GC-MS analysis of glycosyl linkage.

Fig. 3 shows the GC-MS result of the exo-polysaccharide extracted from L24, as an example of all GC-MS studies. It shows the presence of (1→4)-linked-D-glucopyranosyl and (1→6)-linked-D-glucopyranosyl moieties in the glucan, i.e. the peak of 1,4,5-tri-O-acetyl-1-deuterio-2,3,6-tri-O-methyl-D-glucitol with m/z values of 43, 59, 71, 87, 102, 118, 129, 142, 162, 173, and 233 represents glucose (1→4)-linkage and the peak of 1,5,6-tri-O-acetyl-1-deuterio-2,3,4-tri-O-methyl-D-glucitol with m/z values of 43, 59, 71, 87, 102, 118, 129, 143, 162, 173, 189, and 233 represents glucose (1→6)-linkage. Other peaks including 1,4,5,6-tetra-O-acetyl-1-deuterio-2,3-di-O-methyl-glucitol, 1,4,5-tri-O-acetyl-1-deuterio-2,3-di-O-methyl-arabinitol, and 1,2,5-tri-O-acetyl-1-deuterio-3,4,6-tri-O-methyl-mannitol indicate glucose (1→4) and (1→6) linkage, arabinose 1→4 linkage, and mannose 1→2 linkage, respectively (69, 71, 75). The completed GC-MS data are summarized in Table 4.

Based on the above analysis, the raw abundances (i.e. before data normalization) of the five linkage types of all ten *L. edodes* strains are listed in Table 5.

From the data in Table 5, it could be concluded that, for example, the exo-polysaccharide backbone of strain L24 is the longest among all strains because the 2,3,6-Me₃-Glc_p (representing glucose 1→4 linkage) abundance of L24 is the greatest (71). The exo-polysaccharide of L24 has also more glucose 1→4 and 1→6 linkages (represented by 2,3-Me₂-Glc_p abundance) indicating more branches with 1→6 linkage (71). On the other hand, the L24 branches have the least amount of glucose 1→6-linkages because the 2,3,4-Me₃-Glc_p (representing glucose 1→6 linkage) abundance is the least among all. As an other example, the L15 exo-polysaccharide is most likely composed of glucose 1→4 backbone linkage, glucose 1→6 branch linkage, mannose 1→2 branch linkage and arabinose 1→4 branch linkage (75).



The sum of monosaccharide contents of glucose, mannose and arabinose constitutes ~98% of the total monosaccharides for L24. This is consistent with that found from GC-MS linkage data. For other strains, except for L4 with ~73% as the sum, the sums of the glucose, mannose, and arabinose contents are all greater than 90%, and consistent GC-MS results were obtained. These indicate that the glucose 1→4 backbone linkage, glucose 1→6 branch linkage, mannose 1→2 branch linkage and arabinose 1→4 branch linkage are probably dominant in these exo-polysaccharides. However, the significance of these linkage data as related to

strain similarities or differences is not easily decoded by just simple observation and therefore, statistical treatment such as cluster analysis is employed and discussed next.

Cluster analysis.

The results of cluster analysis of the five exo-polysaccharide linkage types employing the normalized abundances data derived from Table 5 of the ten *L. edodes* strains are shown in Fig. 4A, 4B, 4C, 4D and 4E. The relative abundances of all glycosyl linkages in the ten samples were normalized to the myo-inositol internal standard of the L1 sample and were applied for cluster analysis (59). The combination of Euclidean and Manhattan distances with five clustering methods resulted in ten dendrograms. Interestingly, the ten dendrograms form several groupings: one with four identical dendrograms obtained by using the Average, Ward and Weighted linkage type methods of Manhattan distance and using the Ward method of Euclidean distance (Fig. 4A), and another two with two identical dendrograms by using the Average and Weighted methods of Euclidean distance (Fig. 4B) and by using the Complete linkage type method of Euclidean and Manhattan distance (Fig. 4C), respectively. From Fig. 4A, 4B, and 4C, it is observed that L24 either forms an isolated cluster by itself or is close to L15. The Japanese L21, L23, L10, and L11 strains

always form a second cluster with minor differences. The L1, L6, L25, and L4 strains form the remaining clusters with L1 and L25 strains being more similar and L4, and L6 strains being dissimilar. The cluster analysis results using the less common Single linkage method of both Manhattan and Euclidean distances are shown in Supplementary data (Fig. B and Fig. C) which are similar to each other and are somewhat different from those in Fig. 4.

The different hierarchical clustering linkage type methods differ in how the distance between two clusters is computed (87) and resulted in different dendrograms. For example, the average linkage distance between two clusters is defined as the average distance between pairs of objects, one in each cluster (59). Average linkage tends to join clusters with small variances and is biased toward producing clusters with roughly the same variance. In Ward's method, the distance between two clusters is the sum of squares between the two clusters added up over all of the variables (59). Roberts reported Ward's method as "a very efficient clustering method, but favors the grouping of small clusters" (87). Also, Xu suggested that Average linkage method and Ward's method are among the better hierarchical clustering algorithms (59). For our present studies, the cluster analysis utilizing the Ward's method and Average linkage method with both Euclidean and Manhattan distances resulted in two dendrograms (Fig. 4A, 4B). However, these dendrograms are all quite similar, with only the relative

positions of L6 being different. If the Complete linkage type method is considered (Fig. 4C), it is observed that the in addition to L6, the relative position of L15 is different from those of the Average and Ward methods (Fig. 4A and 4B).

The results of the above cluster analysis are not very much different from that of the AFLP assay. The Japanese L10, L11, L21, and 23 strains are both genetically and structurally similar. Other strains show some minor genetic and structural differences. These differences could be attributed to the results of hybridization and other genetic differences.

Conclusion



In addition to the common monosaccharide compositions, molecular mass distributions, and glycosyl linkage information to classify different strains of *L. edodes*, we have carried out AFLP and cluster analyses employing genomic and GC-MS glycosyl linkage data, respectively. The results of both AFLP and cluster analyses provide greater dimensions for mushroom classifications to account for the differences and similarities in their genetic and structural features. Specifically, for cluster analysis, both the Average and Ward methods of Manhattan distance and Euclidean distance seem to be readily useful for this purpose. This approach should be advantageous for more strains or other species classification using valid structural

data. More studies should definitely be carried out to further examine this point.



Tables

Part IV

Table 1. Mycelia dry weight and exo-polysaccharide contents of strains.

Strains	Exo-polysaccharide content (mg/ml)	Mycelia dry weight (g/50ml)
L1	0.58	0.37
L4	0.61	0.4
L6	0.53	0.38
L10	0.31	0.34
L11	0.48	0.4
L15	0.44	0.37
L21	0.2	0.36
L23	0.15	0.33
L24	0.59	0.38
L25	0.59	0.39

Table 2. Molecular mass ratio of exo-polysaccharide from different strains.

Strain/M.W.	L1	L4	L6	L10	L11	L15	L21	L23	L24	L25
Fraction A >2,754,000	0.5	2.5	2.1	3.4	1.7	3.4	2.1	2.5	1.8	3.2
Fraction B 2,754,000	-	0.4	0.4	0.8	-	0.4	0.4	-	0.6	0.7
Fraction C 534,000	1.3	-	-	-	1.8	-	-	0.6	-	-
Fraction D 11,700	1.7	5	1.9	2.2	0.3	0.9	0.7	0.7	5.5	2.8

Table 3. Monosaccharide composition of exo-polysaccharide from different strains.

<i>Lentinula edodes</i>	L1	L4	L6	L10	L11	L15	L21	L23	L24	L25
Glucose %	55.44	23.1	50.95	56.2	47.12	68.93	47.36	56.36	88.82	55.58
Mannose %	32.28	49.22	31.83	26.89	36.24	20.02	33.5	26.72	7.23	30.91
Arabinose %	6.03	11.19	8.25	7.77	6.76	5.41	8.42	7.73	2.22	6.45
Xylose %	4.82	10.65	5.89	5.58	5.21	3.83	5.88	5.49	1.13	4.55
Galactose %	1.44	5.09	2.59	3.56	4.3	1.42	3.73	3.19	0.03	1.87
Fucose %	0	0.52	0.24	0	0.24	0.11	0.56	0.19	0.12	0.24
Rhamnose %	0	0.22	0.24	0	0.14	0.28	0.56	0.32	0.45	0.4

Table 4. GC-MS data from the methylated polysaccharides.

Methylated sugars	Retention time (min)	Mass fragmentation (m/z)	Mode of linkage
2,3,6-Me3-Glcp	21.5	43, 59, 71, 87, 102, 118, 129, 142, 162, 173, 233	¹ Glcp ⁴
2,3,4-Me3-Glcp	21.8	43, 59, 71, 87, 102, 118, 129, 143, 162, 173, 189, 233	¹ Glcp ⁶
2,3-Me2-Glcp	23.9	43, 59, 74, 85, 102, 118, 127, 142, 162, 201, 261	¹ Glcp ^{4,6}
3,4,6-Me3-Manp	21	43, 59, 71, 88, 101, 129, 161, 190	¹ Manp ²
2,3-Me2-Arap	17.8	43, 59, 71, 87, 102, 118, 129, 162, 189	¹ Arap ⁴

Table 5. Methylation analysis of the raw polysaccharide glycosy linkage abundances from different *Lentinula edodes* strains.

Methylated monosaccharides/ Strains	Abundance *10 ⁻⁶					
	Myo-inositol	2,3-Me3-Arap	3,4,6-Me3-Manp	2,3,6-Me3-Glcp	2,3,4-Me3-Glcp	2,3-Me3-Glcp
L1	9.2	2.6	1.4	3.35	0.9	0.9
L4	9.4	0.49	3.43	3.03	1.66	0.88
L6	10.3	0.80	1.52	3.48	1.25	1.52
L10	9.4	0.49	2.15	5.58	1.66	1.86
L11	9.6	1.15	2.40	4.70	1.63	1.63
L15	9.1	2.93	1.72	5.86	1.52	2.43
L21	8.8	1.15	1.78	4.60	1.46	1.46
L23	8.4	0.77	1.42	5.04	2.41	1.53
L24	10.2	0.27	0.09	6.95	0.63	3.25
L25	9.3	0.69	0.10	3.17	1.04	1.09

Figure Captions

Part IV

Fig. 1: AFLP fingerprints of genomic DNAs of different *L. edodes* strains. Two primer sets were used: (left) *EcoR* I -AC-FAM / *Mse* I -CAA and (right) *EcoR* I -AA-FAM / *Mse* I -CAC.

Fig. 2: Dendrogram of *L. edodes* constructed using AFLP assay.

Fig. 3: Gas chromatography-mass spectrometry of L24 exo-polysaccharides.

Fig. 4: (A) Cluster analysis using the Average, Ward and Weighted linkage type method of Manhattan distance and using the Ward linkage type method of Euclidean distance. (B) Cluster analysis using Average and Weighted linkage type method of Euclidean distance. (C) Cluster analysis using the Complete linkage type method of Euclidean and Manhattan distance. (D) Cluster analysis using the Single linkage type method of Euclidean distance. (E) Cluster analysis using the Single linkage type method of Manhattan distance.

PartIV

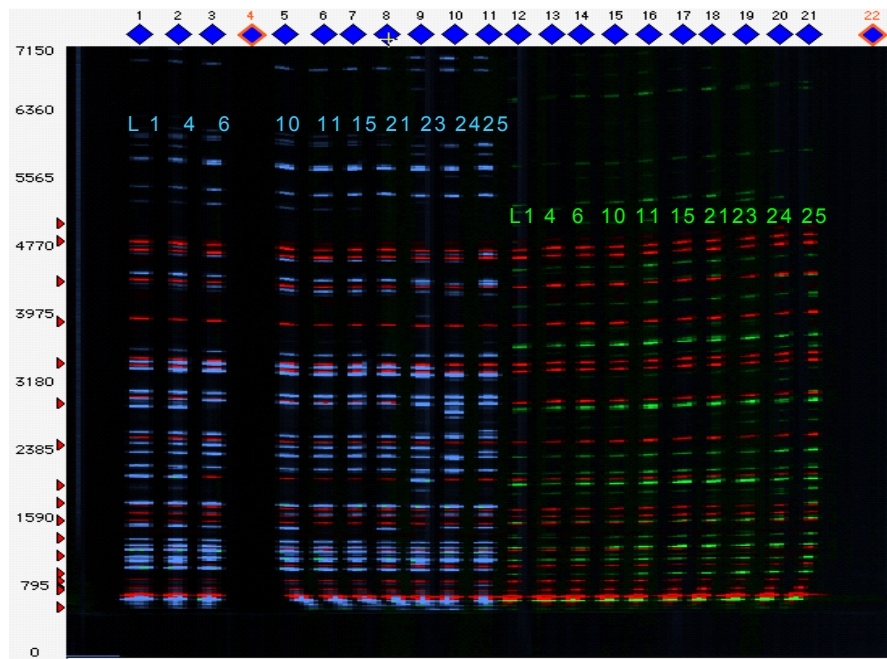


Fig. 1 AFLP fingerprints of genomic DNAs of different *L. edodes* strains. Two primer sets were used: (left) *EcoR* I -AC-FAM / *Mse* I -CAA and (right) *EcoR* I -AA-FAM / *Mse* I -CAC.

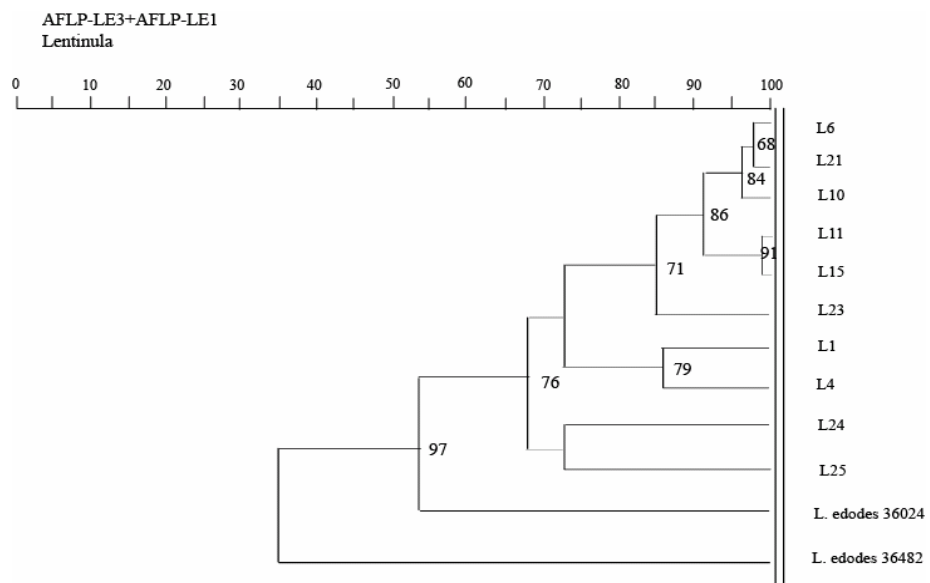
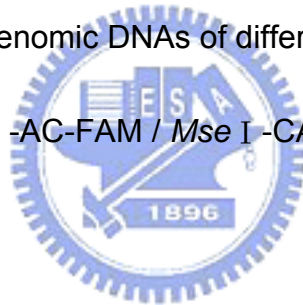


Fig. 2 Dendrogram of *L. edodes* constructed using AFLP assay.

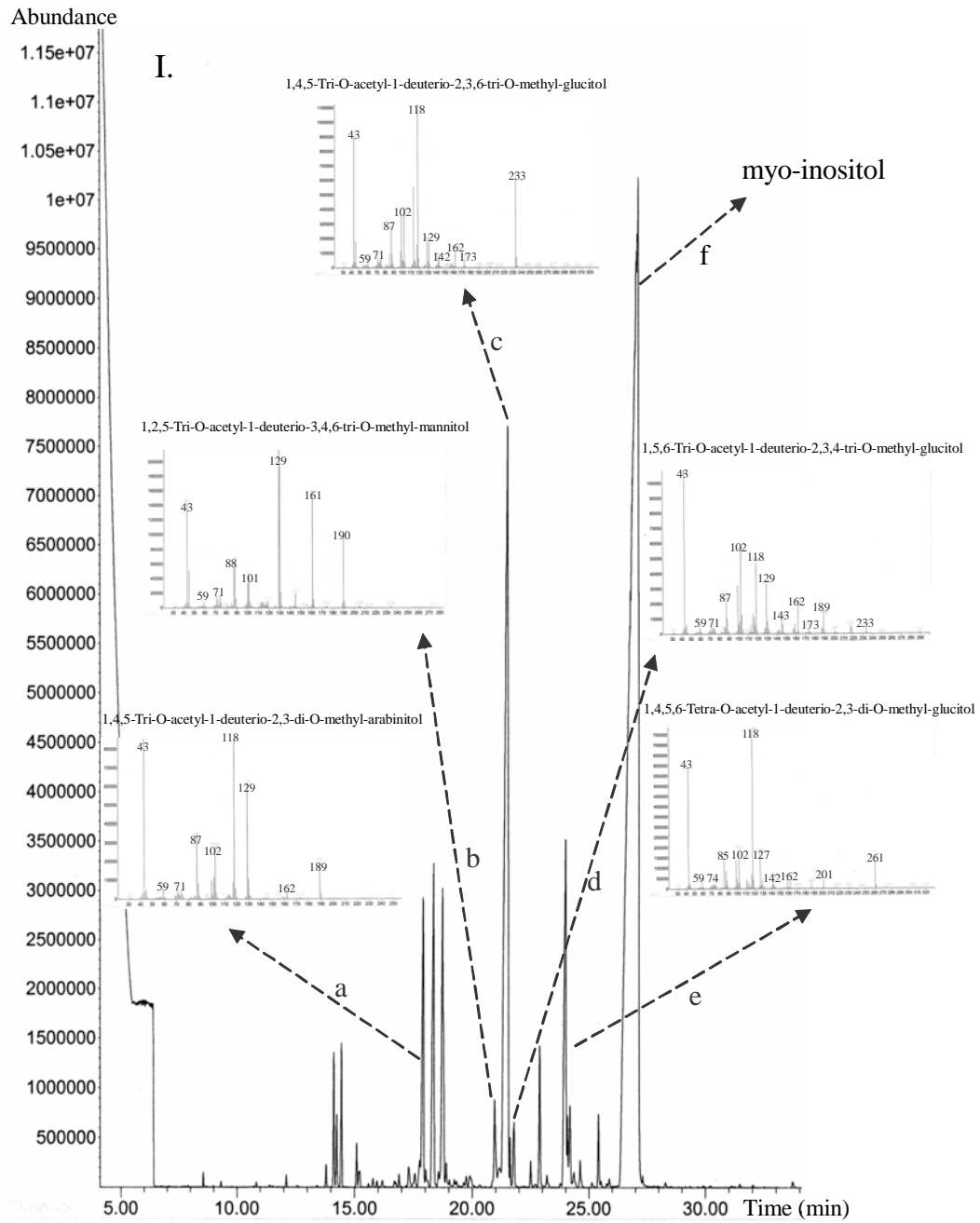
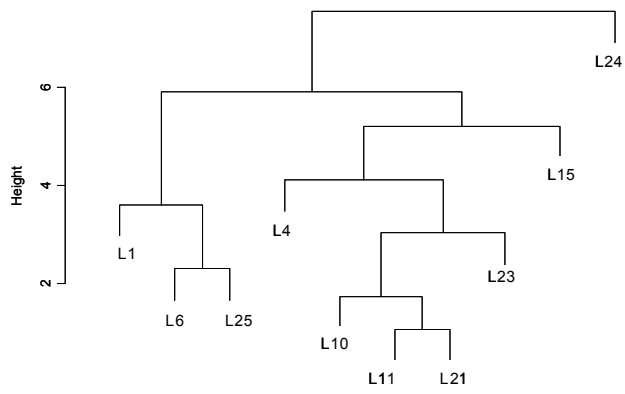
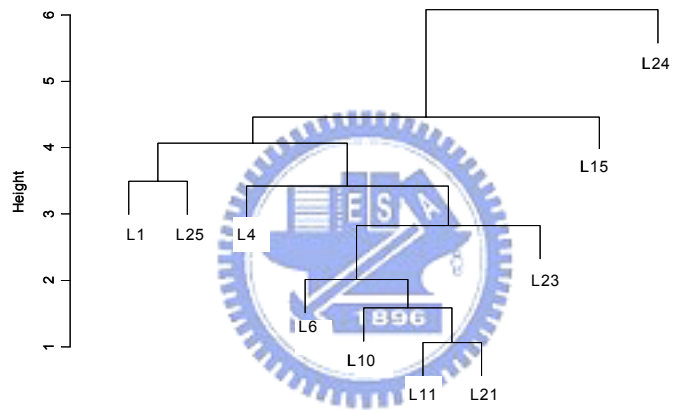


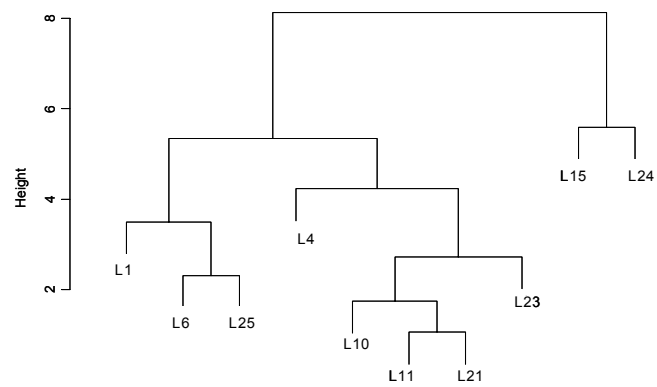
Fig. 3 Gas chromatography-mass spectrometry of L24 exo-polysaccharides.



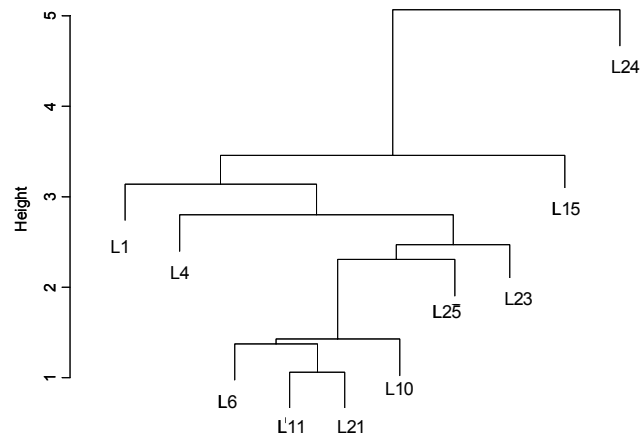
(A)



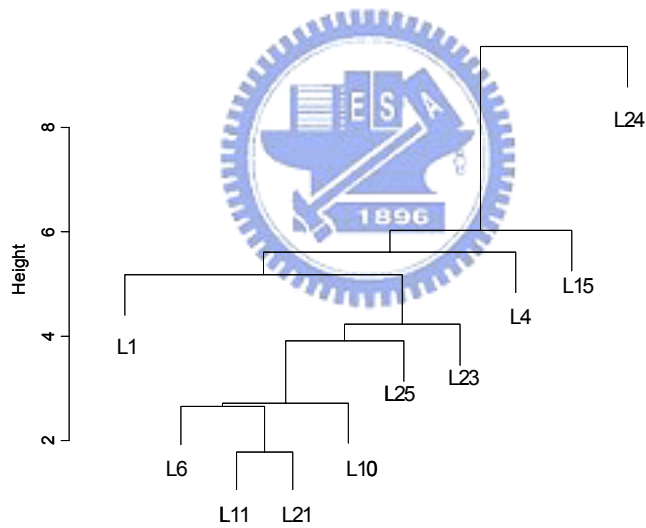
(B)



(C)



(D)



(E)

Fig. 4 (A) Cluster analysis using the Average, Ward and Weighted linkage type method of Manhattan distance and using the Ward linkage type method of Euclidean distance. (B) Cluster analysis using Average and Weighted linkage type method of Euclidean distance. (C) Cluster analysis using the Complete linkage type method of

Euclidean and Manhattan distance. (D) Cluster analysis using the Single linkage type method of Euclidean distance. (E) Cluster analysis using the Single linkage type method of Manhattan distance.



Summary and Future Research Directions

Polysaccharides are known to stimulate macrophage-dependent immune system responses and have been used as complements to conventional cancer therapies widely in Asia for many years and in the western world for the last ten years. A broad range of cells can be activated by polysaccharides, including macrophages, polymorphonuclear leukocytes and natural killer cells. The interaction of polysaccharides with macrophages is generally considered as the first step in the initiation of an immune response. Glucan receptors play an important role in mediating binding of polysaccharides to macrophages. The transcriptional regulation is a major mechanism controlling cytokine expression of macrophage. However, the relationships between polysaccharide structures and signal transduction details of induced macrophage activation are not well understood.

In this thesis, we report our major efforts to isolate exo-polysaccharides from the submerged cultures of ten different strains of *L. edodes* and have determined their structural features and immuno-modulating activities. A new form of polysaccharide linkage with a backbone of (1→4)-glucan and side chains of (1→6)-glucan from *L. edodes* has been identified. In addition, statistical methods including multiple regression, principal component, and cluster analyses have been employed to deduce the polysaccharides structure-function and structure-strain typing

relationships. It was also found that L15 polysaccharides in the presence of LPS could enhance macrophage cellular communications. However, the detailed mechanism at the molecular level remains to be delineated.

Future studies could be designed to answer the following important questions:

1. What are the essential polysaccharide structural features for the macrophage-stimulating activities? What are the bio-active fractions of α -(1→4), β -(1→6)-glucans?
2. What are the potential α -(1→4), β -(1→6)-glucans binding receptors on the macrophages? Are TLR2 or TLR4 involved?
3. What is the signal transduction mechanism of the polysaccharide induced macrophage-stimulating activities? What kinds of macrophage signal transduction factors are activated? What are the roles of NF- κ B signaling pathways in activation macrophage?
4. What are the molecular mechanisms for the α -(1→4), β -(1→6)-glucans or LPS enhanced cell-cell communication phenomenon?

References

1. Reshetnikov, S.V., Wasser, S.P., Tan, K.K., (2001) Higher Basidiomycota as a source of anti-tumor and immunostimulating polysaccharides. *Int. J. Med. Mushrooms*, 3, 361-394.
2. Wasser, S.P., (2002) Medicinal mushrooms as a source of anti-tumor and immunomodulating polysaccharides. *Appl. Microbiol. Biotechnol.* 60, 258–274.
3. Campbell, A.C., Slee, R.W., (1987) Commercial cultivation of Shiitake in Taiwan and Japan. *Mush. J.*, 170, 45-53.
4. Fox, H.M., Burden, J., Chang, S.T., Peberdy, J.F., (1994) Mating Type incompatibility between commercial strains of *Lentinula edodes*. *Exp. Mycology*, 18, 95-102.
5. Hobbs, Ch., (2000) Medicinal values of *Lentinus edodes (berk.) Sing. (Agaricomycetideae)*. A literature review. *Int. J. Med. Mushrooms*, 2, 287-302.
6. Chiu, S.W., Wang, Z.M., Chiu, W.T., Lin, F.C., Moore, D., (1999) An integrated study of individual *Lentinula edodes* in nature and its implication for cultivation strategy. *Mycol. Res.*, 103, 651–660
7. Vos, P., Hogers, R., Bleeker, M., Reijans, M., Lee, T., Hornes, M., Frieters, A., Pot, J., Peleman, J., Kuiper, M., Zabeau, M., (1995) AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res.*, 23, 4407–4414.



8. Geornaras, I., Kunene, N.F., Holy, A. von, Hastings, J.W., (1999) Amplified fragment length polymorphism fingerprinting of *pseudomonas* strains from a poultry processing plant. *Appl. Environ. Microbiol.*, 65, 3828-3833.
9. Jiang, S.C., Louis, V., Choopun, N., Sharma, A., Huq, A., Colwell, R.R., (2000) Genetic diversity of *Vibrio cholerae* in Chesapeake bay determined by amplified fragment length polymorphism fingerprinting. *Appl. Environ. Microbiol.*, 66,140-147.
10. Quagliaro, G., Vischi, M., Tyrka, M., Olivieri, A.M., (2001) Identification of wild and cultivated sunflower for breeding purposes by AFLP Markers. *J Hered.*, 92, 38-42.
11. Savelkoul, P.H.M., Aarts, H.J.M., De Haas, J., Dijkshoorn, L., Duim, B., Otsen, M., Rademaker, J.L.W., Schouls, L., Lenstra, J.A., (1999) Amplified-fragment length polymorphism analysis: the state of an art. *J. Clinical Microbiol.*, 37, 3083–3091.
12. Wang, Z.Y., Tsoi, K.H., Chu, K.H., (2004) Applications of AFLP technology in genetic and phylogenetic analysis of penaeid shrimp. *Biochem. Systematics Ecol.*, 32, 399–407.
13. Terashima, K., Matsumoto, T., Hayashi, E., Fukumasa-Nakai, Y., (2002) A genetic linkage map of *Lentinula edodes* (shiitake) based on AFLP markers. *Mycol. Res.*, 106, 911-917.
14. Terashima, K., Matsumoto, T., (2004) Strain typing of shiitake (*Lentinula edodes*)



- cultivars by AFLP analysis, focusing on a heat-dried fruiting body. *Mycoscience*, 45, 79–82.
15. Mattila, P., Konko, K., Eurola, M., Pihlava, J.M., Astola, J., Vahteristo, L., Hietaniemi, V., Kumpulainen, J., Valtonen, M., Piironen, V., (2001) Contents of vitamins, mineral elements, and some phenolic compounds in cultivated mushrooms. *J. Agric. Food Chem.*, 49, 2343-2348.
16. Mattila, P., Salo-vaananen, P., Konko, K., Aro, H., Jalava, T., (2002) Basic composition and amino acid contents of mushrooms cultivated in Finland. *J. Agric. Food Chem.*, 50, 6419-6422.
17. Bano, Z., Rajarathnam, S., (1988) Pleurotus mushrooms. Part II. Chemical composition, nutritional value, post-harvest physiology, preservation, and role as human food. *CRC Crit. Rev. Food Sci. Nutr.*, 27, 87-158.
18. Crisan, E. V., Sands, A., (1978) Nutritional value. In: Chang, S. T., Hayes, W. A., (eds.). *The biology and cultivation of edible mushrooms*. Academic Press: New York. 251-293
19. Kurzman, R. H., Jr., (1997) Nutrition from mushrooms, understanding and reconciling available data. *Mycoscience*, 38, 247-253.
20. Bohn, J. A., BeMiller, J. N., (1995) (1→3)-β-D-glucans as biological response modifiers: A review of structure-functional activity relationships. *Carbohydr.*

Polymers., 28, 3-14.

21. Chihara, G., Himuri, J., Maeda, Y.Y., Arai, Y. and Fukuoka, F., (1970) Fractionation and purification of the polysaccharides with marked antitumor activity, especially, lentinan, from *L. edodes* (Berk) SING. *Cancer Research.*, 30, 2776-2781.
22. Fujii, T., (1978) Isolation and characterization of a new antitumor polysaccharide, KS-2, extracted from culture mycelia of *Lentinus edodes*, *J. Antibiotics.*, 31, 1079-1090.
23. Harumi S., Akira, O., Sunao, Y., Kenzo, S., Hiroaki, S. Toda. S., (1989) Inhibition of the infectivity and cytopathic effect of human immunodeficiency virus by water-soluble lignin in an extract of the culture medium of mycelia (LEM). *Biochemi. Biophys. Res. Communi.*, 160, 367-373.
24. Suzuki, I, Hashimoto, K, Yadomae, T., (1988) The effects of a highly branched beta-1,3-glucan, SSG, obtained from *Sclerotinia sclerotiorum* IFO 9395 on the growth of syngeneic tumors in mice. *J. Pharmacobiodyn.*, 11, 527-532.
25. Mueller, A., Raptis, J., Rice, P.J., Kalbfleisch, J.H., Stout, R.D., Ensley, H.E., Browder, W., Williams D.L., (2000) The influence of glucan polymer structure and solution conformation on binding to (1→3)-β-D-glucan receptors in a human monocyte-like cell line. *Glycobiology*, 10, 339-346.
26. Aoki, T., (1984) "Lentinan" Immunology studies: Immune modulation agents and

- their mechanisms. Femchel, R.L., and Chirgis, M.A., eds. *Immunology Studies.*, 25, 62-77.
27. Hamuro, J., Wagner, H., Rollinghoff, M., (1978) Beta (1-3) glucans as a probe for T cell specific immune adjuvants. II. Enhanced in vitro generation of cytotoxic T lymphocytes. *Cell Immunol.*, 38, 328-35.
28. Breene, W.M., (1990) Nutritional and medicinal value of specialty mushrooms. *J. Food Protec.*, 53, 883-94, 899.
29. Tabata, T., Watanabe, W., Horita, K., Kamegai, J., Moriyama, S., Hibino, Y., Ohashi, Y., Sugano, N., (1992) Mitogenic activities of heteroglycan and heteroglycan—protein fractions from culture medium of *Lentinus edodes* mycelia. *Immunopharmacology*, 24, 57-63.
30. Mizuno, T., Saito, H., Nishitoba, T., Kawagishi, H., (1995) Anti-tumor-active substances from mushrooms. *Food Rev. Int.*, 11, 23-61.
31. Mizuno, M., Morimoto, M., Minato, K., Tsuchida, H., (1998) Polysaccharides from *Agaricus blazei* stimulate lymphocyte T-cell subsets in mice. *Biosci. Biotechnol. Biochem.*, 62, 434-437.
32. Tanigami, Y., Kusumoto, S., Nagao, S., Koikeguchi, S., Kato, K., Kotani, S. and Shiba, T., (1991) Partial degradation and biological activities of an anti-tumor polysaccharide from rice bran. *Chem. Pharm. Bull.*, 39, 1782-1787.

33. Shida, M., Hargu, K., Matsuda, K., (1975) On the water-soluble heterogalactan from the fruit bodies of *Lentinus edodes*. *Carbohydr. Res.*, 41, 211-218.
34. Shida, M., Uchida, T., Matsuda, K., (1978) A (1→3)- α -D-glucan isolated from the fruit bodies of *Lentinus edodes*. *Carbohydr. Res.*, 60, 117-127.
35. Bao, X., Duan, J., Fang, X., Fang, J., (2001) Chemical modifications of the (1→3)- β -D-glucan from spores of *Ganoderma lucidum* and investigation of their physicochemical properties and immunological activity. *Carbohydr. Res.*, 336, 127–140.
36. Suzuki, T., Ohno, N., Adachi, Y., Cirelli, A. F., Covian, J. A., Yadomae, T., (1991) Preparation and biological-activities of sulfated derivatives of (1→3)- β -D-glucans. *J. Pharmacobio-Dyn.*, 14, 256-266.
37. Willför, S., Sjöholm, R., Laine, C., Roslund, M., Hemming, J., Holmbom, B., (2003) Characterisation of water-soluble galactoglucomannans from Norway spruce wood and thermomechanical pulp. *Carbohydr. Pol.*, 52, 175-187.
38. Zhang, P., Zhang, L., Cheng, S., (2002) Solution properties of an α -(1,3)-D-glucan from *L. edodes* and its sulfated derivatives. *Carbohydr. Res.*, 337, 155-160.
39. Nagi, N., Ohno, N., Adachi, Y., Aketagawa, J., Tamura, H., Shibata, Y., Tanaka, S., Yadomae, T., (1993) Application of limulus test (G pathway) for the detection of

- different conformers of (1→3)-beta-D-glucans. *Biol. Pharm. Bull.*, 16, 822-828.
40. Ohno, N., Asada, N., Adachi, Y., Yadomae, T., (1995) Enhancement of LPS triggered TNF-alpha (tumor necrosis factor-alpha) production by (1→3)-beta-D-glucans in mice. *Biol. Pharm. Bull.*, 18, 126-133.
41. Ooi, E.C.V., Liu, F., (1999) A review of pharmacological activities of mushroom polysaccharides. *Int. J. Med. Mushrooms*, 1, 195-206.
42. Borchers, A.T., Stern, J.S., Hackman, R.M., Keen, C.L., Gershwin, M.E., (1999) Mushrooms, tumors and immunity. *Proc. Soc. Experimen. Biol. Med.*, 221, 281-293.
43. Zhang, L., Chen, L., Xu, X., Lin, Y., Cheung, P.C.K., Kennedy, J.F., (2005) Comparison on chain stiffness of a water-insoluble (1→3)-α-D-glucan isolated from *Poria cocos* mycelia and its sulfated derivative. *Carbohydr. Polym.*, 59, 257-263.
44. Mizuno, T., (1999) The extraction and development of anti-tumor- activities polysaccharides from medicinal mushrooms in Japan (Review). *Int. J. Med. Mushrooms*, 1, 9-29.
45. Rimsten, Lena., (2003) Extractable cell-wall polysaccharides in cereals, with emphasis on β-glucan in steeped and germinated barley. *Doctoral diss. Dept. of Food Science, SLU. Acta Universitatis agriculturae Sueciae. Agraria*. 401.
46. Bhatti, R. S., (1993) Extraction and Enrichment of (1→3),(1→4)-β-D-Glucan from Barley and Oat Brans. *Cereal Chem.*, 70, 73-77

47. Yongguang, Y., Yuzhu, H., Yong, H., (2006) Pulsed electric field extraction of polysaccharide from *Rana temporaria chensinensis* David. *Int. J. Pharm.*, 312, 33-36.
48. Henry, M. C., Yonker, C. R., (2006) Supercritical fluid chromatography, pressurized liquid extraction, and supercritical fluid extraction. *Anal. Chem.*, 78, 3909-3916.
49. Raynie, D. E., (2006) Modern extraction techniques. *Anal. Chem.*, 78, 3997-4004.
50. Piñeriro, Z., Palma, M., Barroso, C. G., (2004) Determination of catechins by means of extraction with pressurized liquids. *J. Chromatogr., A*, 1026, 19-23.
51. Saim, N., Dean, J. R., Abdullah, M. P., Zakaria, Z., (1997) Extraction of polycyclic aromatic hydrocarbons from contaminated soil using Soxhlet extraction, pressurized and atmospheric microwave-assisted extraction, supercritical fluid extraction and accelerated solvent extraction. *J. Chromatogr., A*, 791, 361-366.
52. Barbero, G. F., Palma, M., Barroso, C. G., (2006) Pressurized liquid extraction of capsaicinoids from peppers. *J. Agric. Food Chem.*, 54, 3231-3236.
53. Hartonen, K., Kronholm, J., Riekkola, M. L., (2005) Utilisation of high temperature water in the purification of water and soil. *University of Helsinki Department of Forest Ecology Publications*, 34.

54. Morais, H., Ramos, C., Forgacs, E., Cserhati, T., Oliviera, J., Illes, T., (2001) Three-dimensional principal component analysis employed for the study of the β -glucosidase production of *Lentinus edodes* strains. *Chemom. Intell. Lab. Syst.*, 57, 57-64.
55. Hizukuri, Y., Hashimoto, K., Yamanishi, Y., Kanehisa, M., (2004) Extraction of species-specific glycan substructures, *Genome Informatics*, 15, 69-81.
56. Jin, Y., Zhang, L., Zhang, M., Chen, L., Cheung, P.C.K., Oi, V. E. C., Lin, Y., (2003) Anti-tumor activities of heteropolysaccharides of *Poria cocos* mycelia from different strains and culture media. *Carbohydr. Res.*, 338, 1517-1521.
57. Lo T. C.T., Kang M. W., Wang B. C., Chang A. C., (2007) Glycosyl linkage characteristics and classifications of exo-polysaccharides of some regionally different strains of *Lentinula edodes* by amplified fragment length polymorphism assay and cluster analysis. *Anal. Chim. Acta*, 592, 146-153.
58. Guisan, A., Edwards Jr., T.C., Hastie, T., (2002) Generalized linear and generalized additive models in studies of species distributions: setting the scene. *Ecol. Modell.*, 157, 89-100.
59. Xu, J., Hagler, A., (2002) Chemoinformatics and drug discovery. *Molecules*, 7, 566-600.
60. Mata, G., Delpech, P., Savoie, J.M., (2001) Selection of strains of *Lentinula*

edodes and *Lentinula boryana* adapted for efficient mycelial growth on wheat straw.

Rev. Iberoam. Micol., 18, 118-122.

61. D'souza, F., Garg, A., Bhosle, N.B., (2003) Biogeochemical characteristics of sedimenting particles in Dona Paula Bay. *India*, 58, 311-320.
62. Bermejo, B. P., Moreda, P. A., Bermejo-Barrera A., Bermejo-Barrera A. M., (2002) Application of multivariate methods to scalp hair metal data to distinguish between drug-free subjects and drug abusers. *Anal. Chim. Acta*, 455, 253-265.
63. Hori, R., Sugiyama, J., (2003) A combined FT-IR microscopy and principal component analysis on softwood cell walls. *Carbohydr. Polym.*, 52, 449-453.
64. Mayworm, M.A.S., Buckeridge, M.S., Salatino, A., (2000) Monomer composition of polysaccharides of seed cell walls and the taxonomy of the Vochysiaceae. *Phytochem.*, 55, 581-587.
65. Lo, T. C. T., Jiang, Y. H., Chao, A. L. J., Chang, C. A., (2007) Use of statistical methods to find the polysaccharide structural characteristics and the relationships between monosaccharide composition ratio and macrophage stimulatory activity of regionally different strains of *Lentinula edodes*. *Anal. Chim. Acta*, 584, 50-56.
66. Bouguettaya, A., Viet, L.Q., (1998) Data clustering analysis in a multidimensional space. *Informat. Scien.*, 112, 267-295.
67. Jain, A.K., Murty, M.N., Flynn, P.J., (1999) Data clustering: A review. *ACM*

comput. Surv., 31, 265-323.

68. Vauterin, L.A., Vauterin, P., (1992) Computer-aided objective comparison of electrophoresis patterns for grouping and identification of microorganisms. *Eur. Microbiol.*, 1, 37-41.
69. Blakeney, A.B., Harris, P.J., Henry, R.J., Stone, B.A., (1983) A simple and rapid preparation of alditol acetates for monosaccharide analysis. *Carbohydr. Res.*, 113, 291-299.
70. Hoebler, C., Barry, J.L., David, A., Delort-Laval, J., (1989) Rapid acid hydrolysis of plant cell wall polysaccharides and simplified quantitative determination of their neutral monosaccharides by gas-liquid chromatography. *J. Agric. Food Chem.*, 37, 360-367.
71. Chakraborty, I., Mondal, S., Pramanik, M., Rout, D., Islam, S.S., (2004) Structural investigation of a water-soluble glucan from an edible mushroom, *Astraeus hygrometricus*. *Carbohydr. Res.*, 339, 2249-2254.
72. Kiho, T., Yoshida, I., Nagai, K., Ukai, S., Hara, C., (1989) Polysaccharides in fungi α -(1 \rightarrow 3)-D-glucan from an alkaline extract of *Agrocybe cylindracea*, and anti-tumor-activity of its O-(carboxymethyl)ated derivatives. *Carbohydr. Res.*, 189, 273-279.
73. Bao, X.F., Wang, X.S., Dong, Q., Fand, J.N., Xiao, Y.L., (2002) Structural



- features of immunologically active polysaccharides from *Ganoderma lucidum*.
Phytochem., 59, 175-181.
74. Kim, S.U., Jung, I.C., Kwon, Y.I., Dim, S.Y., Lee, J.S., Lee, H.W., (2000) Characteristics and purification of proteoglycan from *Phellinus igniarius*. *J. Korean Soc. Agri. Chem. Biotechnol.*, 43, 57-62.
75. Capek, P., Hribalova, V., (2004) Water-soluble polysaccharides from *Salvia officinalis* L. possessing immunomodulatory activity. *Phytochem.*, 65, 1983-1992.
76. Komaniecka, I., Choma, A., (2003) Isolation and characterization of periplasmic cyclic β -glucans of *Azorhizobium caulinodans*. *FEMS Microbiol. Lettr.*, 227, 263-269.
77. Tomati, U., Belardinelli, M., Galli, E., Iori, V., Capitani, D., Mannina, L., Viel, S., Segre, A., (2004) NMR characterization of the polysaccharidic fraction from *L. edodes* grown on olive mill wastewaters. *Carbohydr. Res.*, 339, 1129-1134.
78. Rodriguez, A.B., Barriga, C., Lea, R.W., (1996) Effect of prolactin, *in vivo* and *in vitro*, upon heterophil phagocytotic function in the ring dove (*Streptopelia risoria*). *Dev. Comp. Immuno.*, 20, 451-457.
79. Tsumuraya, Y., Misaki, A., (1979) Structure of the water insoluble α -D-glucan of *Streptococcus salivarius* HHT. *Carbohydr. Res.*, 74, 217-225.
80. Tada, H., Shiho, O., Kuroshima, K., Koyama, M., Tsukamoto, K., (1986) An improved colorimetric assay for interleukin-2. *J. Immunol. Methods*, 93, 157.

81. Chihara, G., Hamuro, J., Maeda, Y., Arai, Y., Fukuoka, F., (1970) Fractionation and purification of the polysaccharides with marked antitumor activity, especially lentinan, from *Lentinus edodes* (Berk.) Sing., an edible mushroom. *Cancer Res.*, 30, 2776-2781.
82. Faraji, H., Crowe, T., Besant, R., Sokhansanj, S., Wood, H., (2004) Prediction of moisture content of potash fertilizer using NIR spectroscopy, *Canadian Biosys. Engg.*, 46:3.45-3.48.
83. Hollmen, J., (1996) Principal component analysis. *EET.*, 13, 44:32.
84. Schneeweiss, H., Mathes, H., (1995) Factor analysis and principle components. *J. Multivar. Analy.*, 55, 105-124.
85. Pison, G., Rousseeuw, P., Filzmoser, P., Croux, C., (2001) Robust factor analysis, *J. Multivar. Analy.*, 84, 145-172
86. Sun, K.T., Lai, Y.S., (2002) Applying neural network technologies to factor analysis. *Proc. Natl. Sci. Counc. ROC (D).*, 12, 19-30.
87. Roberts, A., (2002) Automatic acquisition of word classification using distributional analysis of content words with respect to function words. School of computing research report series, School of computing, University of Leeds. *Technical. Report.*
88. Hwang, H.J., Kim, S.W., Choi, J.W., Yun, J.W., (2003) Production and

- characterization of exopolysaccharides from submerged culture of *Phellinus linteus* KCTC 6190. *Enzyme and Microbial tech.*, 33, 309-319.
89. Huang, R.C., Hung, M.L., Wang, B.C., (1999) Phylogenetic study and grouping of commercial shiitake isolates in Taiwan. *J. Chinese Agric. Chem. Soc.*, 37, 431-441.
90. Ahrazem, O., Prieto, A., San-Blas, G., Leal, J.A., Jimenez-Barbero, J., Bernabe M., (2003) Structural differences between the alkali-extracted water-soluble cell wall polysaccharides from mycelial and yeast phases of the pathogenic dimorphic fungus *Paracoccidioides brasiliensis*. *Glycobiology*, 13, 743-747.
91. Grün, C. H., Hochstenbach, F., Humbel, B. M., Verkeij, A. J., Sietsma, J. H., Klis, F. M., Kamerling, J. P., Vliegenthart, J. F.G., (2005) The structure of cell wall α -glucan from fission yeast. *Glocobiology*. 15, 245-257.
92. Chow, J.T-N., Williamson, D.A., Yates, K.M., Goux, W.J., (2005) Chemical characterization of the immunomodulating polysaccharide of *Aloe vera* L. *Carbohydr. Res.*, 340, 1131-1142.
93. Okazaki, M., Adachi, Y., Ohno, N., Yadomae, T., (1995) Structure activity relationship of (1-->3)-beta-D-glucans in the induction of cytokine production from macrophages, *in vitro*. *Biol. Pharm. Bull.*, 18, 1320-1327.
94. Stuelp-Campelo, P.M., Benign, M., De Oliveira, M., Lea, A.M.C., Carbonero,

- E.R., Gorin, P.A.J., Iacomini, M., (2002) Effect of a soluble α -D-glucan from the lichenized fungus *Ramalina celastri* on macrophage activity. *Int. Immunopharmacology*, 2, 691–698.
95. Nair, P.K.R., Rodriguez, S., Ramachandran, R., Alamo, A., Melnick, S.J., Escalona, E., Garcia Jr., P.R., Wnuk, S.F., Ramachandran, C., (2004) Immune stimulating properties of a novel polysaccharide from the medicinal plant *Tinospora cordifolia*. *Int. J. Immunopharmacol.*, 4, 1645–1659.
96. Wang, X.S., Dong, Q., Zuo, J.P., Fang, J.N., (2003) Structure and potential immunological activity of a pectin from *Centella asiatica* (L.) Urban. *Carbohydr. Res.*, 338, 2393–2402.
97. Ukawa, Y., Ito, H., Hisamatsu, M., (2000) Anti-tumor effects of (1→3)- β -D-glucan and (1→6)- β -D-glucan purified from newly cultivated mushroom, Hatakeshimeji (*Lyophyllum decastes* Sing.). *J. Biosci. Bioeng.*, 90, 98-104.
98. Shin, K.S., Kiyohara, H., Matsumoto, T., Yamada, H., (1997) Rhamnogalacturonan II from the leaves of *Panax ginseng* C.A. Meyer as a macrophage Fc receptor expression-enhancing polysaccharide. *Carbohydr. Res.*, 300, 239-249.
99. Knuckles, B.E., Yokoyama, W.H. & Chiu, M.M., (1997) Molecular characterisation of barley β -glucans by size-exclusion chromatography with

- multiple-angle laser light scattering and other detectors. *Cereal Chem.*, 74, 599-604.
100. Beer, M.U., Wood, P.J. & Weisz, J., (1997) Molecular weight distribution and (1→3), (1→4)-β-glucan content of consecutive extracts of various oat and barley cultivars. *Cereal Chem.*, 74, 476-480.
101. Yap, A. T., Ng, M. L. M., (2001) An improved method for the isolation of lentinan from the edible and medicinal shiitake mushroom, *Lentinus edodes* (Berk.) Sing. (Agaricomycetidae). *Int. J. Med. Mushrooms*, 3, 6-19.
102. Suortti, T., Gorenstein, M. V., Roger, P., (1998) Determination of the molecular mass of amylose. *J. Chromatogr. A*, 828, 515–521.
103. Han, J. A., Lim, S. T., (2004) Structural changes in corn starches during alkaline dissolution by vortexing. *Carbohydr. Pol.*, 55, 193-199.
104. Wasser, S. P., (2002) Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. *Appl. Microbio. Biotechnol.*, 60, 258-274.
105. Daba, A. S., Ezeronye, O. U., (2003) Anti-cancer effect of polysaccharides isolated from higher basidiomycetes mushrooms. *African J. Biotechnol.*, 2, 672-678.
106. Akassoglou, K., Adams, R. A., Bauer, J., Mercado, P., Tseveleki, V., Lassmann, H., Probert L., Strickland, S., (2004) Fibrin depletion decreases inflammation and delays the onset of demyelination in a tumor necrosis factor transgenic mouse model for multiple sclerosis. *PNAS*, 101, 6698-6703.

107. Cleary, J. A., Kelly, G. E., Husband, A. J., (1999) The effect of molecular weight and β -1,6-linkages on priming of macrophage function in mice by (1,3)- β -D-glucan. *Immun. Cell. Biol.*, 77, 395-403.
108. York, S. W., (1995) A conformational model for cyclic β -(1 \rightarrow 2)-linked glucans based on NMR analysis of the β -glucans produced by *Xanthomonas campestris*. *Carbohydr. Res.*, 278, 205-225.
109. Koizumi, K., Sanbe, H., Kubota, Y., Terada, Y., Takaha, T., (1999) Isolation and Characterization of cyclic α -(1 \rightarrow 4)-glucans having degrees of polymerization 9-31 and their quantitative analysis by high-performance anion-exchange chromatography with pulsed amperometric detection. *J. Chromatogr. A*, 852, 407-416.
110. Chow, J. T-N., Williamson, D. A., Yates, K. M., Goux, W. J. (2005) Chemical characterization of the immunomodulating polysaccharide of *Aloe vera* L. *Carbohydr. Res.*, 340, 1131-1142.
111. An, J., Carlson, R. W., Glushka, J., Streeter, J. G., (1995) The structure of a novel polysaccharide produced by *Bradyrhizobium* species within soybean nodules. *Carbohydr. Res.*, 269, 303-317.
112. Mueller, U.G., Wolfenbarger, L.L., (1999) AFLP genotyping and fingerprinting. *Tree*, 14, 389-394.

