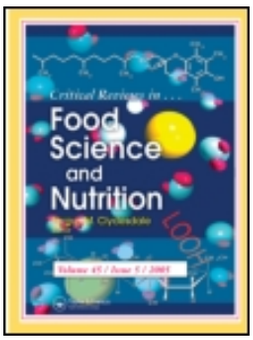

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Polysaccharides in *Lentinus edodes*: Isolation, Structure, Immunomodulating Activity and Future Prospective

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Lentinus edodes *has been valued as edible and medical resources. Polysaccharides have been known to be the most potent antitumor and immunomodulating substance in* Lentinus edodes. *In this review, we summarize the current knowledge of the polysaccharides isolated from* Lentinus edodes, *including extraction and purification methods, chemical structure and chain conformation, the effects on innate and adaptive immunity and their mechanism, relationship between structure and function, and the future prospects.*

Keywords *Lentinus edodes*, polysaccharide, lentinan, immunomodulation, antitumor

INTRODUCTION

Lentinus edodes (Berk.), named as Shiitake in Japanese, has been cultivated for thousands of years. Due to its excellent availability in food and folk medicine, *L. edodes* is now one of the most popular edible mushrooms in East Asia. Dried *L. edodes* contains carbohydrates (58–60%), proteins (20–23%), fiber (9–10%), lipids (3–4%), and ash (4–5%) (Wasser, 2003). As a foodstuff, it is appetizing and nourishing. As a medicinal material, it has many pharmacological activities, including antifungal/antibacterial, antiviral, immunomodulatory, and antitumor activities (Bisen et al., 2010). Among several substances responsible for these bioactivities, polysaccharides are well studied. Lentinan, a β-1,3-glucan, is the most important polysaccharide isolated from *L. edodes*, because of its immunomodulatory and antitumor effects. This paper focuses on the current understanding of polysaccharides especially lentinan from *Lentinus edodes* with respect to extraction, purification, structure, immunomodulatory activities, and mechanism.

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ISOLATION METHOD AND PURIFICATION PROCEDURE OF POLYSACCHARIDES

Mushroom polysaccharides exist as a structural component of the cell wall. The cell wall of mushroom contains two main polymers, chitin and β-glucan. The individual chains in both molecules are linked with hydrogen bridges so that covalent bonds form between the two polymers. This process results in the formation of a strong cell wall, in which chitin fibers are interlinked with glucan to create a network matrix (Otakar et al., 2009). The cell wall structure of the fruit body of *L. edodes* can be divided into three layers: (1) the outside layer is heteropolysaccharide and β-(1→3)-glucan with β-(1→6) branches, which can be extracted by water and diluted alkali solution; (2) the middle layer is mainly the β-(1→6)-glucan with a small number of β-(1→3) branches, which are water insoluble and can be extracted out by strong alkali solution; and (3) the inner layer is a complex of chitin, β-glucan, and a small amount of acid polymer (Shida et al., 1981).

The extraction method can be varied with the structure and water solubility of polysaccharides. The basic rule is to break the cell wall from the outer layer to the inner layer with mild-to-strong extraction conditions. Extracted polysaccharides can be further purified with a series of combined techniques, such as

precipitation with ethanol, acetic acid, or cetyltrimethylammonium bromide; protein removal with Saveg segment; decolorization by H_2O_2; ion-exchange chromatography; gel filtration; and affinity chromatography. Generally, ion-exchange chromatography through diethylaminoethyl cellulose columns can separate neutral polysaccharide from acid ones (Wasser, 2002). There are three kinds of basic extracting solvents: acid solution, alkali solution, and water. Water extraction method is a popular approach. By now, several kinds of polysaccharides have been identified from *L. edodes* with different extraction methods and purification processes (see Table 1). The chemical structures of polysaccharides isolated from *L. edodes* vary with extraction methods and sites of fruit body.

STRUCTURE AND PROPERTIES

Physical Properties

Lentinan refers to a highly purified polysaccharide fraction extracted from the fruit body of *L. edodes* (Chihara et al., 1970). The physical properties of lentinan are the following: $[\alpha]_D^{20}$+19.5 to +21.5°(10%NaOH), insoluble in cold water, and slightly soluble in hot water (Chihara et al., 1970). Another report indicated that lentinan is β-D-(1,3)-D-glucopyranan with a branched chain of β-D-(1,6)-monoglycosyl, is water soluble, and has the properties of $[\alpha]_D$+20 to +22°(NaOH) (Wasser, 2005). The slightly different physical properties may be attributed to the slightly different structure in side chains.

Chemical Structure

In the past four decades, the most in-depth study was conducted on structure and activity of lentinan. Sasaki and Takasuka have demonstrated that the chemical structure of lentinan has a main chain consisting of β-D-(1→3)-linked D-glucopyranosyl residues with two (1→6)-β-glucoside branches for every five D-glucose residues, and the side chains of lentinan consist of β-D-(1,6)-linked and β-D-(1→3)-linked glucose residues (Sasaki and Takasuka, 1976). Its average molecular weight was first determined by light scattering measurement to be 9.5×10^5–10.5×10^5 Da (Maeda and Chihara, 1971) and later found to be 3×10^5 to 8×10^5 Da by gel permeation chromatography and quasielastic light scattering measurement (Suzuki et al., 1982). It seems that only high molecular weight (>90,000 Da) molecules can form triple-helix structures, which are stabilized by the β-D-glucopyranosyl branch units (Saito et al., 1977, 1991).

Conformation Transition

It has been proved that lentinan exists as a molecule with a triple-helix conformation in water and NaCl solution, but it adopts a random coil in dimethyl sulfoxide (DMSO) and urea solution (Maeda et al., 1988; Zhang et al., 2001, 2002). For example, the values of molecular weight (M_w) for lentinan in 0.5 M NaCl aqueous solution are about three times of those in DMSO. This suggests that lentinan exists as a triple-chain structure in an aqueous solution, whereas it dissociates into a single-chain structure in DMSO (Zhang et al., 2001). The triple-helix structure of lentinan is formed by the intramolecular and intermolecular hydrogen bonds. The disruption of the intramolecular and intermolecular hydrogen bonds leads to the conformation transition. So, the conformation transition of lentinan molecule remarkably depends on the solvent, the temperature, and the pH in the solution (Zhang et al., 2001, 2002, 2004; Wang, Xu, et al., 2008).

The property of the solvent determines whether the intramolecular and intermolecular hydrogen bonds form or not. Zhang et al. (2002) have found that lentinan exhibited an irreversible transition from triple-helix to single coil conformation when the water composition decreased to about 0.15 (W/W) in a mixture of water and DMSO at 25°C. Interestingly, chain collapse and aggregation of lentinan molecules have been observed in DMSO solution when water is added to the DMSO solution from 0 to 100% (Xu et al., 2004). The rheological properties of lentinan in solution can be referred to in the review by Zhang, Li, Wang, et al. (2011)).

Temperature is an important factor affecting the conformation of lentinan in solution. The helix–coil transition induced by thermal treatment has been studied, showing the irreversible conformation transition from triple-helix to single coil at about 130–145°C (Wang, Xu, et al., 2008). When temperature is favorable for the formation of triple-helix, the cyclic and linear triple-helix structures can be formed, depending on polymer concentration and thermal history (McIntire and Brant, 1998). In DMSO/water mixture solution, the chain structure of lentinan exhibits two transitions with increasing temperature (Wang et al., 2009). The first transition appears at low temperature (under 45°C) from a highly ordered triple-helix (triple-helix I) to a disordered one (triple-helix II). The transition is due to the changes in the hydrogen bonding between water and the side chain of lentinan. The second transition appears at higher than 80°C from triple-helix II to single coil, which resulted from the destruction of the intramolecular and intermolecular hydrogen bonds in lentinan. The conformation transition temperature depends on the concentration of DMSO (Wang et al., 2009). In pure water, the first transition appears at bout 7–9°C for lentinan (Zhang et al., 2008).

The alkali concentration affects the degree of formation and destruction of intramolecular and intermolecular hydrogen bonds in solution. In alkali solution, the helix–coil transition can be observed in different concentrations of the alkali solution. Saito et al. (1977) reported that lentinan keeps an ordered conformation up to a concentration of 0.2 M sodium hydroxide. Near 0.19 M sodium hydroxide, lentinan exists as a single-helix conformation. By atomic force microscopy, viscometry, multi-angle laser-light-scattering, and optical rotation measurements,

Table 1 Identified polysaccharides isolated from *L. edodes*

Sample name	Main extraction method and purification procedure	Properties of polysaccharide	References
Lentinan (isolated from the fruit body of *L. edodes*)	Extracted with hot water, precipitated with ethanol, CTAB, the precipitate be treated with acetic acid, NaOH, and removal of protein by Sevag method, and then dried.	β-(1→3)-D-glucan with (1→6) branches, M_w: 4∼8 × 10^5, do not contain nitrogen, sulfur, phosphorus, carbohydrate content is 99.23%, slightly water soluble.	Chihara et al., 1969, 1970; Yap and Ng, 2001
L-II (isolated from the fruit body of *L. edodes*)	Extraction by distilled water, concentration, removal of protein by Sevag method, precipitation with ethanol, purified by DEAE-cellulose, and lyophilization.	β-glucan, consisted of glucose, M_w: 2.03 × 10^5, carbohydrate content is 90.14%.	Zheng et al., 2005
Lentinan (isolated from the fruit body of *L. edodes*)	Homogenization, freezing with liquid nitrogen, lyophilization, extraction with water, precipitation with ethanol, and lyophilization.	Carbohydrate content is 87.65% and residue has 11% protein and 1% fat.	Yap and Ng, 2001
L-FV-I(lentinan) (isolated from the fruit body of *L. edodes*)	Treatment with EtOAc and MeOH, extraction with 0.9% NaCl and hot water in sequence. Residue was extracted with 5% NaOH/0.05% $NaBH_4$. Precipitates were separated by adjusting to pH 7 in supernatant. The supernatant was precipitated with ethanol and purified by dialysis and Sevag method and then dried.	β-(1,3)-D-glucan with (1,6) branch linkages, M_w: 7.79 × 10^5, contain 10% of protein, water soluble.	Zhang et al., 1999; Zhang et al., 2002
Heterogalactan (isolated from the fruit body of *L. edodes*)	Extraction with 3% trichloroacetic acid, followed by precipitation with methanol.	A main chain of α-(1→6)-D-galactopyranose, parts of which are substituted in 2-position with fucose or mannose, water soluble.	Shida et al., 1975
FMG (heterogalactan) (Isolation from the fruit body of *L. edodes*)	Extraction with cold water, followed by precipitation with EtOH, then further purified with Cu^{2+} precipitation, ion-exchange risins, ultrafiltration, and lyophilization.	A heterogalactan with a main chain of (1→6)-linked α-D-galactopyranosyl units, partially substituted at O-2 by single-unit D-Man*p* or L-Fuc*p* side chains, M_w: 16.2 × 10^3.	Carbonero et al., 2008
α-D-glucan (isolated from the fruit body of *L. edodes*)	Treated with 3% trichloroacetic acid and hot water, residue was extracted with sodium hydroxide, neutralization of supernatant, reticulation in alkali, and reprecipitation in acetic acid.	α-(1→3)-D-glucan, a slightly branched structure composed of (1→3)- and (1→4)-linked, water insoluble.	Shida et al., 1978
L-FV-II(α-D-glucan) (isolated from the fruit body of *L. edodes*)	Defatted with EtOAc and MeOH, 0.9% NaCl, and hot water in sequence. Residue was extracted with 5% NaOH/0.05% $NaBH_4$. Precipitation by adjusting to pH 7 in supernatant. Precipitates were purified by dialysis and Sevag method, and then dried.	α-(1→3)-D-glucan with slight (1→6) branch linkages, M_w: 2.41 × 10^5, water insoluble.	Zhang et al., 1999
Lew (isolated from the cap of the fruit body of *L. edodes*)	Extraction with hot water, precipitated with ethanol and CTAB, removal of protein with Sevag method, dialyzed with water, and lyophilization.	β-heteropolysaccharide. carbohydrate content is 79.1%. M_w: 2.03 × 10^4, Ara:Rha:Xyl:Gal:Glc = 4.36:2.03:1.00:5.64:13.80.	Zhang and Lu, 1998
Lea (isolated from the cap of the fruit body of *L. edodes*)	Extraction with alkali solution, precipitated with ethanol and CTAB, removal of protein with Sevag method, dialyzed with water, and lyophilization.	β acid heteropolysaccharide, carbohydrate content is 83.2%, M_w: 9.40 × 10^4, Ara:Rha:Xyl:Gal:Glc = 9.37:1.00:1.64:7.19:23.36.	Zhang and Lu, 1998
PJFI (isolated from the stipe of the fruit body of *L. edodes*)	After being treated with SLS, PAW, DMSO, the stipe extracted with hot water, purified by Sepharose several times, and then lyophilization.	β-(1→3)-linked backbone with (1→6)-linked side chain, Xyl:Man:Glc:Gal = 5:6:75:5.	Zheng and Geng, 1995
KS-2 (isolated from the mycelium of *L. edodes*)	Extraction of cultured mycelia of shiitake mushroom (strain KSLE 007) with hot water, followed by precipitation with ethanol.	An α-mannan peptide containing the amino acids serine, threonine, alanin, and praline, M_w: 6∼9.5 × 10^4.	Ishida et al., 1979; Wasser, 2005
LE (isolated from the mycelium of *L. edodes*)	Extraction with water, precipitates with ethanol followed by centrifugation and washed with acetone, dialyzed and applied to DEAE-cellulose, elucted with 0.01M Tris-HCl buffer, and lyophilization.	β-(1,3)-D-glucan with β-(1,6)-D-glucose side chains, M_w: 5.08 × 10^5 Da, containing 94.2% carbohydrate and 5.8% protein.	Liu et al., 1998
Compound X (isolated form the mycelium of *L. edodes*)	Extraction with water, precipitation with ethanol, precipitates redissolved with water and precipitate with 0.2M CTA-OH (cetytrimothylammonium hydroxide). After being treated with acetic acid, the precipitates were dissolved in NaOH solution, followed by dialyzed and freeze-dried.	β-xylan, containing xylose, ribose, arabinose, and mannose. Xylose is present large amount. M_w: 2–3.5 × 10^5 Da.	Tomati et al., 2004

Note: CTAB, cetyltrimethylammonium bromide; DEAE-cellulose, diethylaminoethyl cellulose; SLS, Sodium lauryl sulfate; PAW: Phenol-acetic acidwater.

lentinan exists as triple-helix chains and as single random-coil chains in aqueous sodium hydroxide with the concentration lower than 0.05 M and higher than 0.08 M, respectively (Zhang et al., 2004). Lentinan molecules in a 0.12 M borate buffer, pH = 10, are random coil chains (Suzuki et al., 1982). This indicated that lentinan exists with three kinds conformations (triple-helix, single-helix, and random coil) subject to the concentration of sodium hydroxide or pH. When the concentration of NaOH is below 0.05 M, lentinan exists as a triple-helix conformation. Irreversible helix–coil transition occurred rapidly at an NaOH concentration between 0.05 and 0.08 M. The triple-helix conformation cannot be reversed by acid neutralization. However, the denatured lentinan can be reassociated to triple-helix conformation by dialyzing against abundant water (Zhang et al., 2004). These results were similar to the earlier results (Maeda et al., 1988), but the concentration of NaOH causing conformation transition is different. These results also indicated that the structure of lentinan was slightly different in different experiments.

IMMUNE MODULATION AND ANTITUMOR ACTIVITIES OF LENTINAN

Mushroom polysaccharides cause no harm and place no additional stress on the body; therefore, they are regarded as biological response modifiers (BRMs). The proposed mechanism by which mushroom polysaccharides exert antitumor effect include (1) the prevention of the oncogenesis (cancer-preventing activity), (2) enhancement of immunity against the bearing tumors (immune modulation activity), and (3) direct antitumor activity by inducing the apoptosis of tumor cells (direct tumor inhibition activity) (Zhang et al., 2007). Lentinan is considered as a T-cell-oriented immunopotentiator and, therefore, requires a functioning T-cell component for its biological activities. The action of β-(1→3)-glucan on the host's immune system might be threefold: (1) increased T helper (Th) cell production, (2) increased macrophage production, and (3) a nonimmunological increase of host defense mechanisms through stimulated acute phase proteins and colony-stimulating factor (CSF), which in turn affects the proliferation of macrophages, peripheral mononuclear cells, and lymphocytes and the activation of the complement system (Bohn and BeMiller, 1995).

Cancer Prevention Activity

The cancer-preventive activity of medical mushroom polysaccharides has been observed first in farmers whose main occupation was producing medical mushroom. These farmers' cancer death rate was remarkably lower than that of the general population by 40% (Ikekawa, 2001). Some efforts have been made to unravel the possible reasons. The cytochrome P450s (CYP) is a class of drug and xenobiotic-metabolizing enzymes mainly expressed in the liver, and the suppression of CYP activity is considered to prolong the duration of and intensify drug action. This action contributes to the prevention of carcinogenesis, because the CYP1A subfamily, which is induced by xenobiotics such as polyaromatic hydrocarbons, metabolically activates procarcinogens to their ultimate forms (Hashimoto et al., 2002). Lentinan prepared from *L. edodes* was intraperitoneally injected to female BALB/c mice, and the effects of polysaccharide on the expression of CYPs were investigated in the liver. Lentinan downregulated the activity and level of constitutive and 3-methylcholanthrene-inducible CYP1A accompanied by the TNF-α (tumor necrosis factor-alpha) production through the suppression of DNA-binding activity of aryl hydrocarbon receptor and an increase in the DNA-binding activity of nuclear factor-κB (Hashimoto et al., 2002; Okamoto et al., 2004). These results suggest that the mushroom polysaccharides such as lentinan have an anticarcinogenic activity, since the downregulation of CYP1A is considered to prevent the metabolic activation of procarcinogens (Hashimoto et al., 2002; Okamoto et al., 2004). In addition, the prevention of carcinogenesis may also be related to the inhibition of telomerase activity by lentinan (Sreenivasulu et al., 2011). Lentinan not only is useful for cancer treatment as an immunopotentiator in combination with anticancer drug, but also prevents the increase of chromosomal damage induced by anticancer drug in vivo. Research revealed that the extracts from the fruit body of *L. edodes* inhibit mutagenicity of CP (cyclophosphamide) and ENU (N-ethyl-N-nitrosourea) in vivo (Lima et al., 2001).

Immune Modulation Activity

The previous studies showed that lentinan does not attack cancer cells directly, but produces their antitumor effects by activating different immune responses in a host (immune modulation activity). Lentinan can cause over 90% reduction in tumor size or complete regression in most of the tested animals (Chihara et al., 1969, 1970). It demonstrated antitumor activity not only against allogeneic tumors, but also against various synergic and autochthonous tumors (Wasser, 2002). The antitumor activity of lentinan was lost in neonatal thymectomized mice (mice have no thymus-dependent immune system) and decreased significantly by the administration of antilymphocyte serum (Maeda and Chihara, 1971). The results showed that the antitumor activity requires an intact T-cell component and that the activity is mediated through thymus-dependent immune mechanism. The antitumor activity of lentinan is inhibited by pretreatment with antimacrophage agents (such as carrageenan). In addition, lentinan is able to restore the suppressed activity of Th cells in the tumor-bearing host to their normal state, leading to the complete restoration of humoral immune responses (Maeda et al., 1988). The administration of lentinan can promote potentiation of response of precursor T cells and macrophages to cytokines produced by certain groups of lymphocytes

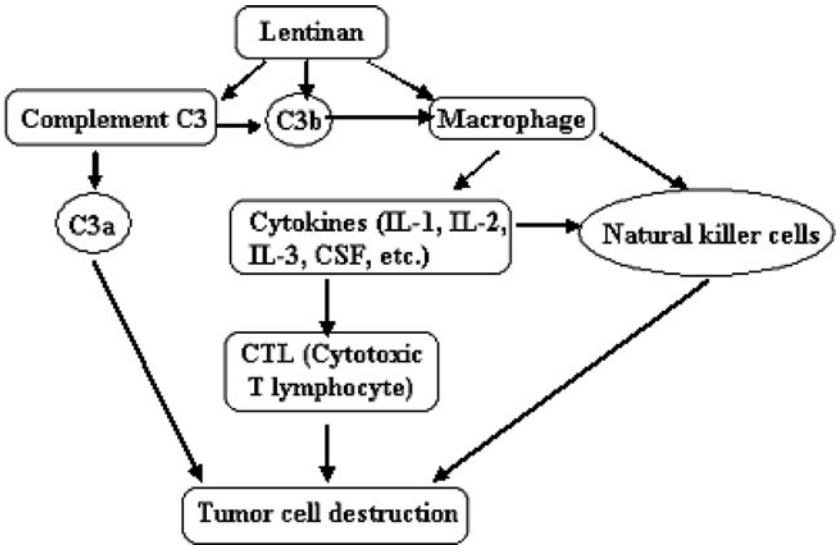

Figure 1 Possible mechanism of antitumor activity of lentinan.

after specific recognition of tumor cells (Chihara, 1992). The induction of a marked increase in the amounts of TNF-α, IL-1 (interleukin-1), IL-3 (interleukin-3), and IFN (interferon) by lentinan results in maturation, differentiation, and proliferation of immunocompetent cells for host defense mechanisms (Chihara, 1992). However, the effectiveness of lentinan to induce cytotoxicity and TNF secretion was highly influenced by genotype of the host (Kerekgyarto et al., 1996). Moreover, it has been reported that the delayed-type hypersensitivity response induced at tumor sites by lentinan, and the subsequent infiltration of immune effect cells, such as natural killer cells and cytotoxic T lymphocytes (CTLs), is an important mechanism of antitumor action for lentinan (Suzuki et al., 1994). It has been proposed that lentinan inhibits hepatic metastasis in adenocarcinoma-26-bearing mice by activating Kupffer cells (Taki et al., 1995). Infiltration of eosinophils, neutrophils, and granulocytes around target tissues is also accelerated by lentinan. It activates secretion of active oxygen and production of cytokines in peritoneal macrophages. Lentinan also increases peritoneal macrophage cytotoxicity against metastatic tumors; it can activate the normal and alternative pathways of the complement system and can split complement component 3 (C3) into C3a and C3b, enhancing macrophage activation (Wasser and Weis, 1997; Hobbs, 2000; Wasser, 2002). The possible mechanism of antitumor activity of lentinan has been summarized (Chihara, 1992) and later reviewed (Wasser and Weis, 1999; Wasser, 2002) (see Figure 1). The pathway explained the possible immune mechanism of lentinan. It remains to be clarified that immunomodulatory effects induced by lentinan are critical for tumor rejection. In addition, lentinan also exhibits antiinflammatory effects on gut inflammation and oral ulceration (Mizuno et al., 2009; Yu et al., 2009), the detailed reason may be due to the suppression of IL-8 mRNA (messenger ribonucleic acid) expression through TNF-α (Mizuno et al., 2009).

Direct Tumor Inhibition Activity

Direct tumor inhibition activity has been documented in many mushroom polysaccharide studies (Wang et al., 2002). Some studies indicated that incubation of polysaccharides together with tumor cells could change the expression of signals within the tumor cells, affect cell cycle, and generate apoptosis, which explains in vitro the antiproliferation effects of polysaccharides (Chen and Chang, 2004; Li et al., 2004). These results suggest that mushroom polysaccharides not only stimulate T lymphocytes and the immune function through the immune modulation, but also have a direct action on the tumor cells. For example, lentinan from the fruit body of *L. edodes* inhibits the proliferation of Sarcoma 180 (S-180) cells in vitro and the inhibition ratio is related to the molecular weight of lentinan (Zhang et al., 2005). Sia and Candlish (1999) have reported that the aqueous extract from the fruit body of *L. edodes* can induce apoptosis in human neutrophils and U937 cells. Also, the aqueous extracts from both the fruit body and mycelia of *L. edodes* can directly inhibit the proliferation of MCF-7 (Michigan Cancer Foundation-7) human breast adenocarcinoma cell line. These results demonstrated that the extracts from *L. edodes* have the direct cytotoxic activity on tumor cells (Israilides et al., 2008). The direct cytotoxic activity may be due to the polysaccharides or polysaccharide-peptide complex isolated from *L. edodes*. Little is known about the direct effect of polysaccharides on cancer cells. Recent studies on direct cytotoxicity of polysaccharides have indicated that polysaccharides can directly inhibit the cancer cell proliferation in a dose- and time-dependent manner through upregulation of p21 and downregulation of cyclin D1 (Zaidman et al., 2005). One additional point to note is that the purified degree of lentinan, which varies with the applied extraction method and purification process (see Table 1), may affect the results in different experiments in vivo and in vitro.

Highly purified and defined structures of polysaccharide from *L. edodes* are needed to investigate the detailed direct tumor inhibition mechanism.

POTENTIAL MECHANISM OF ACTION

Chemical structure characteristics have demonstrated that lentinan is a typical fungal β-glucan. Current studies suggest that fungal β-glucan is a potent immunomodulator with effects on both innate and adaptive immunity (Kupfah et al., 2006). The innate immunity is superior to and has an instructive role on the adaptive immunity due to the reason that cells and molecules of the innate immune system have the ability to distinguish between self and non-self to elicit tailor-made response by activating different effectors; however, the adaptive immunity is completely opposite in that they are not having any specialization before activation (Wong et al., 2011). So, the adaptive immunity is regulated by the innate immune system (Iwasaki and Medzhitov, 2010).

Possible Receptors and Signaling Pathway for Innate Immune Modulation

It is proved that there are fungal pattern-recognition molecules for the innate immune system. Innate immune pattern-recognition receptors play critical roles in pathogen detection and initiation of antimicrobial responses. The mechanism by which the innate immune system recognizes and responds to fungal cell wall β-glucan is a multipathway and multifactorial process (see Figure 2). Fungal β-glucan acts on several immune receptors on immune cells including Dectin-1 (Dennehy and Brown, 2007; Taylor et al., 2007), complement receptor type 3 (CR3) (Ross et al., 1987), toll-like receptor (TLR-2/4/6) (Chan et al., 2009; Ronald and Beutler, 2010), lactosylceramide (LacCer) (Zimmerman et al., 1998), and scavenger (Rice et al., 2002), and triggers a group of immune cells including macrophages, neutrophils, monocytes, natural killer cells, and dendritic cells (DCs). In particular, the mechanism of signal transduction via receptor–ligand interactions remains unclear. Dectin-1 has been described as an important β-(1→3)-glucan receptor, because it is essential for (1) the production

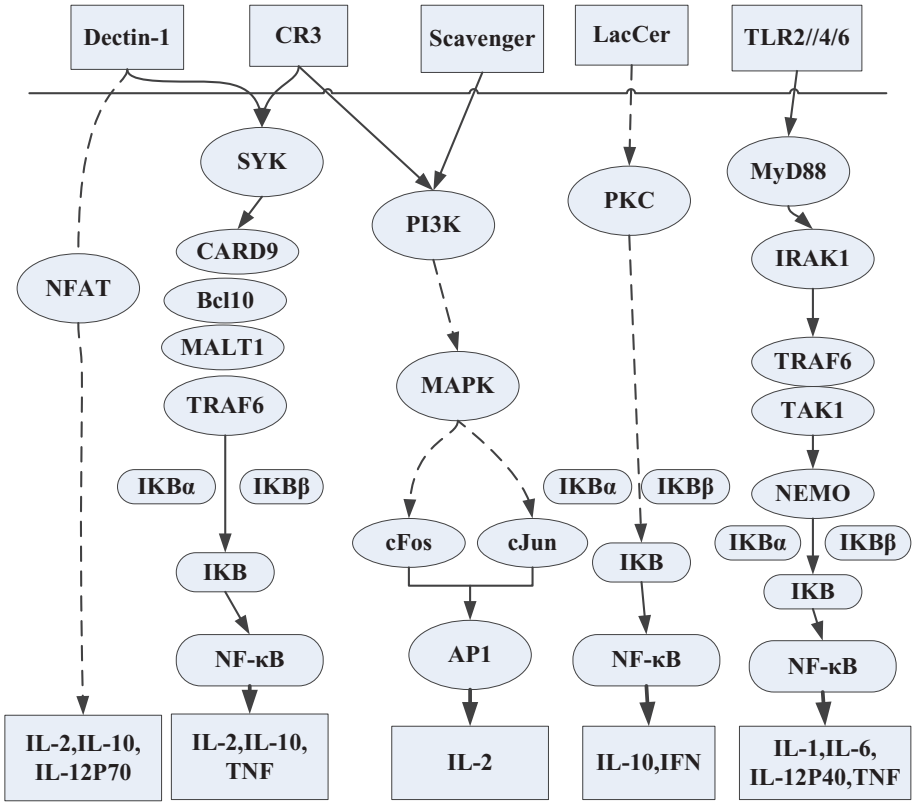

Figure 2 The innate immunity response signaling pathway in immune cells activated by β-glucan. It should be noted that different receptors likely cooperate with each other (e.g., Dectin-1-TRL2) forming signaling complexes to induce many responses. Abbreviation: CR3, complement receptor type 3; LacCer, lactosylceramide; TLR, toll-like receptor; NF-κB, nuclear factor kappa B; NFAT, nuclear factor of activated T cells; NEMO, NF-κB essential modulator; IKK, inhibitor of NF-κB kinase; PKC, protein kinase C; PI3K, phosphoinositide-3-kinase; MAPK, mitogen-activated protein kinase; IRAK1, IL-1R-associated kinase; MyD88, myeloid differentiation protein 88; TRAF6, TNF-receptor-associated factor 6; JNK, c-Jun N-terminal kinase; AP1, activator protein 1; IκB, inhibitor of NF-κB; IKK, IκB kinase; SYK, spleen tyrosine kinase; CARD9, capase recruitment domain 9; Bcl10, B-cell lymphoma 10; MALT1, mucosa-associated lymphoid tissue 1; TNF, tumor necrosis factor. (Color figure available online.)

of cytokines and reactive oxygen species by both DCs and macrophages after stimulation with β-(1→3)-glucan and (2) the host defense response against fungi (Brown and Gordon, 2001; Adachi et al., 2004; Saijo et al., 2007; Taylor et al., 2007). Studies reveal that β-glucan induces Dectin-1-mediated signal transduction via spleen tyrosine kinase (Syk) and CARD/Bcl10 pathways (Brown, 2006; Gross et al., 2006; LeibungGut-Landmann et al., 2007). Tada et al. (2009) have demonstrated that the binding of barley-derived β-glucan to Dectin-1 triggers the signal transduction and stimulates the production of NF-κB (nuclear factor kappa B) leading to the cytokine production when Dectin-1, Syk,CARD9, and Bcl10 are coexpressed in the cells. Although being dispensable for TLR/MyD88-induced responses, CARD9 controls Dectin-1-mediated myeloid cell activation, cytokine production, and innate antifungal immunity. CARD9 couples to Bcl10 and regulates Bcl10–Malt1-mediated NF-κB activation induced by β-glucan (Gross et al., 2006).

Dectin-1 signals alone are sufficient to trigger phagocytosis and Src–Syk-mediated induction of antimicrobial reactive oxygen species (Underhill et al., 2005); collaboration with TLR signaling enhances NF-κB activation and regulates cytokines production (Gantner et al., 2003; Huang et al., 2009). Furthermore, Dectin-1 signaling can also directly modulate gene expression via activation of nuclear factor of activated T cells (NFAT). The role of NFAT activation has been defined most comprehensively in T cells: thymocyte development, T-cell differentiation, T-cell activation, and anergy are all regulated by NFAT (Macian, 2005). NFAT activation regulates IL-2, IL-10, and IL-12p70 production in macrophages and DCs by β-glucan stimulation. At the same time, NFAT combination with AP1 can synergistically activate IL-2 transcription (Nguyen et al., 2010). These establish NFAT activation in myeloid cells as a novel mechanism of regulation of the innate antimicrobial response (Goodridge et al., 2007).

CR3 is another important receptor for complement (C3b)-opsonized particles, β-glucan, and microbial particles (Ross, 2000). It is reported that lentinan is capable of activating a complement system and inhibits tumor growth via alternative pathway (Lull et al., 2005). Researches have shown that β-glucan-mediated CR3-dependent cytotoxicity is greatly decreased by the inhibition of signaling molecules: phosphorylation of the tyrosine kinase, Syk, and consequent PI3K (phosphatidylinositol-3 kinase) (Li et al., 2006). Thus, β-glucan enhances tumor killing through a cascade of events, including in vivo macrophage cleavage of the polysaccharide, dual CR3 ligation, and CR3-Syk-PI3K signaling pathway. In addition to CR3, CR1 would be another possible receptor for lentinan. However, the binding of lentinan to human monocytes differs in individuals (Oka et al., 1995). Lentinan also has been found to bind to scavenger receptor on the surface of myeloid cells thus triggering PI3K, Akt kinase, and p38 mitogen-activated protein kinase signaling pathway (Lin et al., 2005).

Some chemical molecules affect the innate immune responses. For example, β-glucan's immunostimulating signaling pathway is related to the Ca^{2+} in the immunity cells. Calcium is an important second messenger that plays a key role in signaling T-lymphocyte activation. An increase in Ca^{2+} concentration and subsequent protein kinase C activation is indispensable for T-lymphocyte proliferation. It has been demonstrated that lentinan can improve the concentration of cytosolic-free Ca^{2+} on splenocytes. Zymosan was reported to increase lymphocyte Ca^{2+} concentration by promoting influx of extracellular Ca^{2+} and increasing intracellular Ca^{2+} release (Chen et al., 2003). Lentinan promotes chemosensitivity in colon carcinoma cells by inducing calcium-sensing receptor expression in the human colon carcinoma cells (Wang, Chen, et al., 2010). Lentinan can also stimulate H_2O_2 and NO production by macrophages and markedly enhances the innate immune response (Markova et al., 2002, 2003). For example, lentinan can improve killing ability against *Mycobacterium tuberculosis* and *Staphylococcus aureus* of alveolar macrophages (Drandarska et al., 2005).

The Effects on Adaptive Immune Modulation

The adaptive immune system functions through the combined action of antigen-presenting cells (APCs) and T cells. β-glucan acts as antigen to play a role in the first step of activation signaling pathway in APCs (Cobb et al., 2004). In view of the fact that certain different receptors exist on the APCs and Th cells can recognize and interact with antigen-major histocompatibility complex (MHC) II molecule; various cytokines are produced that affect the activation of B cells, Tc cells, macrophages, and other T cells, and hence influence inflammation, antimicrobial, and antitumor activities (see Figure 3).

β-(1→3)-D-glucans were identified almost 40 years ago as BRMs that stimulated tumor rejection. Previous reports had shown that there was a marked deregulation in the balance between Th1 (T helper cell type 1) and Th2 (T helper cell type 2) immune response in the course of cancer progression, demonstrating the dominant Th2-type responses as a consequence of the progressive loss of Th1-type responses in tumor-bearing animals or cancer patients (Maeda and Shiraishi, 1996; Pellegrini et al., 1996; Tabata et al., 1999; Yoshino et al., 2000). Because Th1 cells enhance the CTL response while Th2 cells suppress it, the cytokine patterns, which reflect the Th1/Th2 balance, are crucial in mediating resistance to cancer. Cancer immunotherapy that abolishes this Th2-dominant response and promotes instead a Th1 response would improve cancer resistance. The effects of β-glucan on Th1 and Th2 immune responses have been evaluated. Polysaccharide extracted from *L. edodes* can augment the expression level of IL-2 and TNF-α in vitro, which induces the production of IL-2 and TNF-α (Liu et al., 1998). Intravenously administered lentinan can cancel a Th2-dominant condition in patients with digestive tract cancer and improve the balance between Th1 and Th2 (Yoshino et al., 2000).

Subsequently, the detailed process has been investigated. In vivo, lentinan elicited peritoneal macrophages to reduce the release of IL-10 and IL-6, while it endowed macrophages with the elevated capability to produce IL-12 and nitric oxide (NO)

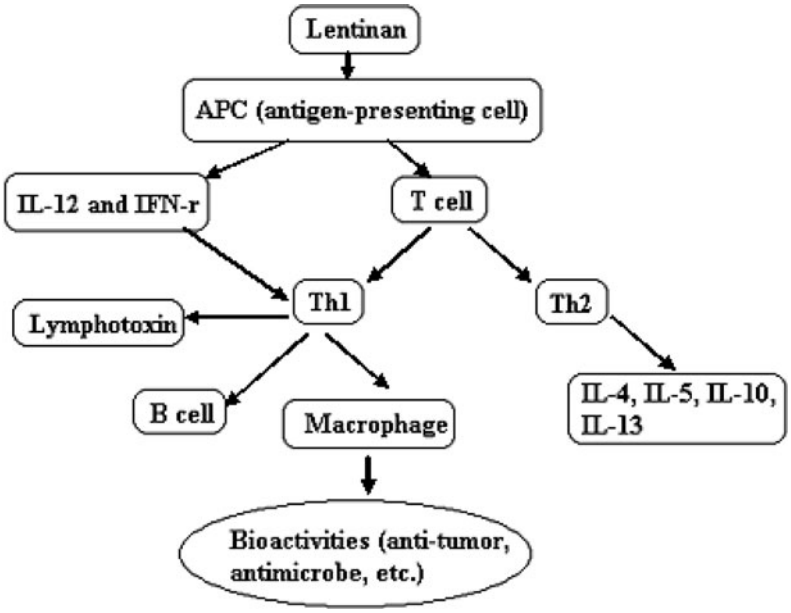

Figure 3 Schematic representation of the affecting of immunomodulatory on the adaptive immune system activated by lentinan.

upon in vitro triggering, due to the elevated intracellular glutathione (GSH) content in macrophages. Deprivation of intracellular GSH completely ablated the production of IL-12 (Murata, Shimamura, Tagami, et al., 2002). The IL-2 administration in combination with lentinan exerted the synergistic augmentation of IL-12 and NO and reduction in IL-6 production. It was also confirmed that CD4+T cells derived from lentinan-administered mice showed augmented IFN-γ and reduced IL-4 production upon anti-CD3 stimulation in vitro (Murata, Shimamura, Tagami, et al., 2002). This indicated that skewing of Th1/Th2 balance to Th1 by lentinan is directed through the distinctive production of IL-12 versus IL-6, IL-10 by macrophages, depending on intracellular GSH redox status (Murata, Shimamura, Tagami, et al., 2002; Murata, Shimamura, and Hamuro, 2002; Yamada, 2009). For the efficient tumor immunotherapy, maybe one of the critical elements is to induce a reductive form of macrophages in tumor stromal tissues to maintain Th1 response. In vivo, lentinan induces Th1 cell development and plays an important role in maintaining the balance between Th1 and Th2 response (Murata, Shimamura, and Hamuro, 2002). In addition, the intracellular cytokine profiles of peripheral blood lymphocytes from mice bearing mammary tumors, which received intravenously antitumor monoclonal antibodies combined or not with whole β-glucan particle suspension given orally, were assessed. The proportions of T cells producing IL-4 and IFN-γ were determined by flow cytometry. The proportion of T cells producing IL-4 was significantly higher in tumor-bearing mice not receiving β-glucan-enhanced therapy. Conversely, T cells from mice undergoing β-glucan-enhanced therapy showed increased production of Th1 cytokine IFN-γ (Baran et al., 2007). The results indicated that the switch from a Th2 to a Th1 response was possibly mediated by intestinal mucosal macrophages releasing IL-12 (Baran et al., 2007). Furthermore, lentinan can result in enhanced expression of MHC II, CD80/CD86, and TLRs (TLK2/TLK4), and increased production of IL-12 in spleen DCs (Zhou et al., 2009). These results indicated that lentinan induces Th1 immune response on adaptive immune response.

The Gastrointestinal Tract Absorption Mechanism of β-Glucan in vivo

Abundant evidence demonstrates the ability of β-(1→3)-glucan to activate macrophages and neutrophils when given intravenously or intraperitoneally. Numerous studies also showed that the activities of β-glucan were maintained with oral administration (Ng and Yap, 2002; Vetvicka et al., 2002, 2007; Rice et al., 2005; Baran et al., 2007; Demir et al., 2007). However, it has been a longstanding puzzle about how effective the orally administered β-glucan is. It has been speculated that the immunomodulatory properties of β-glucan may be partly attributed to a microbial-dependent effect because most of β-glucans are considered as nondigestible carbohydrates and are fermented by the intestinal microbial flora (Ohno et al., 1995; Wang, Weening, et al., 2008).

In order to reveal the absorption mechanism, experiments were carried out. After oral administration, it was found that the specific backbone 1→3 β-glycosidic chain of β-glucan cannot be digested. Researchers have labeled β-glucan with fluorescein on the reducing terminus to track their oral administration and processing in vivo. β-glucan enters the proximal small intestine and some are captured by the gastrointestinal macrophages. They are internalized and fragmented within the cells and then transported by the macrophages to the marrow and endothelial reticular system. The small β-glucan fragments are eventually

released by the macrophages and taken up by other immune cells leading to various immune responses (Hong et al., 2004; Chan et al., 2009). For example, β-glucan of 150 kDa generated a fragment with an approximate molecular size of 25 kDa, which appears to bind to CR3 on neutrophils and mediates antitumor cytotoxic effects (Li et al., 2006).

In addition, other researchers found that soluble β-glucans (glucan phosphate, laminarin, and scleroglucan) after oral administration were bound and internalized by intestinal epithelial cells and gut-associated lymphoid tissue (GALT) cells. Internalization of glucan by intestinal epithelial cells was not Dectin-1 dependent, but Dectin-1 and TLR 2 account for the increasing uptake of soluble glucan by GALT cells (Rice et al., 2005).

As for lentinan, oral administration routes, intrapleural, intraperitoneal, or intravenous, have been studied (Chan et al., 2009). In aqueous solution, the particle size of lentinan is approximately 100–200 μm; this impedes the absorption of lentinan particles through the intestinal mucosa (Yamada et al., 2007). After oral administration of the dispersed lentinan (superfine dispersed β-1,3-glucan), a special treatment of lentinan is confirmed to be adhesed onto and uptaked into Peyer's patches of the small intestine and electron microscopy showed that the lentinan particles were present in the vacuoles of epithelial cells (Suga et al., 2005).

RELATIONSHIP BETWEEN STRUCTURE AND IMMUNOMODULATING ACTIVITY

Effects of Primary Chemical Structure on Bioactivity

The primary structure of polysaccharide includes the sequence of monosaccharide in backbone chain and side chain, degree of branch, side group, and glycosidic linkages. Lentinan debranching side groups by Smith degradation is still effective against Sarcoma 180 (Sasaki and Takasuka, 1976). This indicates that the side groups of C1-6 glycosidic linkage glucose have little influence on their antitumor activity. Sulfated derivation of lentinan with single flexible chain had a lower inhibitory effect on the growth of S-180 than the native ones, but had a significant increase compared with the denatured lentinan having single flexible chain formation. This indicates that the introduction of sulfated groups has benefited the bioactivity of denatured lentinan by increasing the water solubility and chain stiffness (Wang and Zhang, 2009). Furthermore, the sulfated lentinan has better antiviral activity than nonmodified lentinan (Wang, Guo, et al., 2010). These results also suggest that the contribution for triple-helix conformation of lentinan to its bioactivity is more important than the side groups.

However, some researchers pointed out that the activity of lentinan is dependent on its basic structure, oligosaccharide unit, rather than its macroscopical morphology (Ning et al., 2003). Oligosaccharides about β-(1,6)-branched β-(1$\rightarrow$3) glucohexaose and its analogs containing an α-(1,3)-linked bond have been synthesized to investigate the activity. The results suggest that the synthetic oligosaccharides have similar stimulatory effects on the mouse spleen as lentinan (Ning et al., 2003; Yan et al., 2003) and can enhance the virus-specific CTL and Th1 responses (Wang, Dong, et al., 2010).

Under certain circumstances, the biological efficiency of β-glucan can be promoted by the presence of their complexes with proteins, for example, highly active immune-stimulating glycoproteins, called fungal immunomodulatory proteins (Otakar et al., 2009). Glucan with helix shows a strong ability to attach itself to some proteins and to form a complex, which then stimulates production of antibodies by macrophages (Adachi et al., 1999). Lentinan with bound protein exhibits higher antitumor activity than that without bound protein in vivo (Surenjav et al., 2006). Lentinan composed of 11% proteins, which is prepared from the modified procedure, shows antitumor property in vivo. However, no antitumor properties were observed for the separated proteins from lentinan with further purification procedure (Yap and Ng, 2001; Ng and Yap 2002). These may suggest that the degree of structure complexity is associated with the potency of immunomodulating and antitumor activities.

Effects of Molecular Weight on Bioactivity

The molecular weight of polysaccharides is a factor affecting the bioactivity. Molecular weight can affect their ability to interact with the surface of leukocytes (Yadomae, 2000). The solubility of β-glucan is known to be related to its molecular weight. Lentinan is insoluble in water, but "small lentinan" ($M_w > 16{,}000$ g/mol) prepared by hydrolysis with formic acid is soluble, which also showed antitumor activity (Sasaki and Takasuka, 1976; Saito et al., 1977). Recently, the fractions of lentinan with different molecular weights have been prepared by ultrasonic irradiation to degrade the polysaccharide in aqueous solution in Zhang's group. Lentinan with different M_w from 28.3×10^5 g/mol to 3.57×10^5 g/mol have been achieved by ultrasonic treatment. The triple-helix lentinan with a moderate molecular weight (5.0×10^5 to 15.0×10^5 g/mol) exhibits higher antitumor activities than those with too low or too high molecular weight. Data show that the polysaccharide with maximum inhibition ratio against S-180 was lentinan with M_w of 11.4×10^5 g/mol in vivo and 5.71×10^5 g/mol in vitro (Zhang et al., 2005). The activity differences among lentinans with different molecular weights may be due to the proportion of the ordered aggregates with the triple-helix chain in the aqueous solution (Zhang, Li, and Zhang, 2010). The result demonstrated that the antitumor activity of lentinan is related to the molecular weight indeed, but the relationship between M_w and the activity is different in vivo and in vitro.

The molecular weight of polysaccharide also affects the particle size and the clinical quality at last. A double-blind, placebo-controlled randomized study is designed in which participants received a daily oral dose of superfine dispersed lentinan (SDL) and nondispersed lentinan (NDL) for 2 months, and the effects on allergic rhinitis and rhinoconjunctivitis were analyzed

(Yamada et al., 2007). The results showed that the oral uptake of SDL reduced allergic symptoms and NDL produced no clinical effects. The mean diameters of SDL and NDL are 0.08 μm and 288 μm, respectively (Shen et al., 2007; Yamada et al., 2007). The SDL can be taken up by Peyer's patches of the small intestine and is present in the vacuoles of epithelial cells (Suga et al., 2005). Another multicenter clinical study in patients with advanced colorectal cancer was conducted to investigate the safety and effectiveness of SDL. The results showed that SDL was safe and effective for suppressing the adverse effects of chemotherapy as well as improving quality of life (Hazama et al., 2009). These indicate that the clinical quality was correlated with the molecular size of β-glucan: small particles pass through the intestinal mucosa more easily than the larger ones.

Effects of Chain Conformation on Bioactivity

A triple-helix conformation of medical mushroom β-(1→3)-glucan is known to be important for the immunostimulating activity. When lentinan was denatured with DMSO, urea, or sodium hydroxide, the triple-helix structure was lost while the primary structure was not affected, but tumor inhibition activity of denatured lentinan was lower than that of undenatured lentinan (Maeda et al., 1988). Similar results have been obtained by other researchers (Zhang et al., 2005). Sulfated derivatives of lentinan with single flexible chains in solution still have antitumor activity (S-180), but lower than those of the triple-helix lentinan (Wang and Zhang, 2009). These results demonstrate the relationship between antitumor activity and triple-helix structure of lentinan. However, it is still unclear how the triple-helixl conformation of β-(1→3)-glucan affects their antitumor activity. Some of the immunopharmacological activities such as macrophage nitrogen oxide synthesis and limulus factor G activation are dependent on the triple-helix conformation, while synthesis of IFN-γ and CSF is independent of the triple-helix conformation (Zhang et al., 2007).

FUTURE CHALLENGE AND PERSPECTIVES

Polysaccharides belong to a structurally diverse class of macromolecules, in which monosaccharide units are joined to each other by glycosidic linkages. The monosaccharide units within polysaccharides can interconnect at several points to form a wide variety of branched or linear structures. So, just like the other natural materials, a polysaccharide cannot be 100% purely prepared and does not have a constant molecular weight, which may affect the clinical quality. Due to the high molecular weight and poor solubility in water, it is hard to extract pure lentinan from the fruit body of *L. edodes*. Studies have illustrated that the protein bound to lentinan, which was isolated from the fruit body of *L. edodes*, exhibits important effects in immune responses in vivo (Yap and Ng, 2001; Ng and Yap 2002; Surenjav et al., 2006). The detailed reasons need further investigation in the future.

Recent studies of the interaction between β-glucan and its corresponding receptors on human immune cells focus on β-glucan from yeast. However, β-glucans from different resources with different chemical structure characteristics have different receptors and show different binding affinity to the same receptor. Furthermore, β-glucans with different structure characteristics may have different signaling pathways (Muller et al., 2000; Schepetkin and Quinn, 2006). It is envisaged that new receptor(s) might be found in human immune cells for mushroom polysaccharides including lentinan. Investigation of the receptor-mediated pathways for the immunomodulatory activity by lentinan is needed. On the other hand, it seems that a nonlinear relationship exists between the dosages of lentinan used and its activities. For example, lentinan caused complete regression of S-180 transplanted into imprinting control region mice at a dose of 1 mg/kg for 10 days, while a large dose of 80 mg/kg for 5 days showed no antitumor activity in comparison with untreated control mice (Chihara et al., 1969, 1970; AoKi, 1984). Thus, lentinan exhibits a dose-dependent manner in antitumor activity and should have an optimal dosage; the detailed mechanism deserves to be investigated. In conclusion, the biochemical mechanisms of lentinan that act within the body are still insufficient; further research is required to investigate the biochemical mechanism.

Different polysaccharides isolated from *L. edodes* vary with the extraction methods and sites of the fruit body (see Table 1). Furthermore, lentinan isolated from different strains of *L. edodes* with same extraction method and purification process shows differences in molecular weight, protein content, and antitumor activity (Sugui et al., 2003; Surenjav et al., 2006). So, a carefully appropriate extraction method with good quality control and lentinan standard should be well defined in order to understand the mechanism on how lentinan acts on the immune system. Moreover, only about 5% of lentinan is obtained from the fruit body of *L. edodes* by frequently used extraction methods (Wang, Xu, et al., 2008). However, β-glucan, the predominant component in the cell wall of *L. edodes*, nears to 65% (Shida et al., 1975, 1978). It means that there is a large space to improve the yield of lentinan. New method for isolating polysaccharide from *L. edodes* should be developed in the future. For example, high yield of Sparan (70.2%) and Phellin (65.2%) with remarkably high water solubility have been obtained by nanonknife method (Park et al., 2009). At the same time, a highly efficient purification process also needs to be developed (Jiang et al., 2009).

The structure of polysaccharide is so diverse that it is difficult to define the precise structure. Classic analysis methods are time–cost and the structure information obtained from the analysis methods is complicated (Dell and Morris, 2001; Zhang et al., 2007). By now, the knowledge about the structure–function relationship of lentinan is still limited. A future challenge lies in putting together all the elementary structure parameters determined by all kinds of instrumental methods, using computer-assisted methods, to form a three-dimensional (3D) structure of polysaccharides and illustrate the structure–function

relationship. This can provide a good opportunity for scientists to design high-bioactivity drugs based on the 3D structure. In future work, it will be important to determine structure–function relationships and define specific structure contributing to optimal bioactivity properties. Also, highly efficient analysis methods should be developed to accelerate the progress of polysaccharide structure research.

Animal model and clinical studies have demonstrated that lentinan exhibits a number of beneficial therapeutic properties, including immunostimulatory (Chihara, 1992), antitumor (Chihara et al., 1969, 1970), antiallergic symptoms (Yamada et al., 2007; Yamada, 2009) and anti-inflammation (Mizuno et al., 2009; Yu et al., 2009). Lentinan can increase the effectiveness of chemotherapeutic preparations/vaccine leading to reducing their side effects (Suzuki et al., 1990; Drandarska et al., 2005; Guo et al., 2009), thus improving life quality and prolonging the lifetime of cancer patients (Nakayama et al., 2004; Nimura et al., 2006; Yang et al., 2008; Hazama et al., 2009; Kataoka et al., 2009). Lentinan in combination with IL-2 or other therapies induces synergistically immune responses (Hamuro et al., 1994; Murata, Shimamura, and Hamuro, 2002; Murata, Shimamura, Tagami, et al., 2002) and is beneficial in terms of increasing survival duration, tumor necrosis, and preventing tumor metastases (Sano et al., 2002; Yang et al., 2008; Hori et al., 2011). Lentinan also shows positive effects on intestinal health. It can increase the resistance of intestinal mucosa to inflammation (Zeman et al., 2001; Mizuno et al., 2009) and inhibit the development of intestinal ulcers (Nosalova et al., 2001). However, technical ways are necessary to improve their clinical qualities, water solubility, and ability to permeate the gastrointestinal mucosa after oral administration, such as chemical modification, enzyme modification, or nanobiotechnology. In particular, the detailed mechanism of action for lentinan is not yet sufficient; extensive research using novel omics technologies (e.g. transcriptomics, proteomics, and metabonomics) is highly required.

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