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Review

Antitumor polysaccharides from mushrooms: a review on their isolation process, structural characteristics and antitumor activity

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Mushrooms have been valued as edible and medicinal resources, and antitumor substances have been identified in many mushroom species. Polysaccharides are the best known and most potent mushroom-derived substances with antitumor and immunomodulating properties. Although the isolation process, structural characterization and antitumor activity of mushroom polysaccharides have been extensively investigated in the past three decades, the relationship between the antitumor activity and the chemical composition as well as the high order structure of their active components is still not well established. These studies are still in progress in many laboratories, and the role of polysaccharides as antitumor agent is especially under intense debate. The purpose of the present review is to summarize the available information, and to reflect the current status of this research area with a view for future direction.

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Introduction

In Asian countries like China and Japan, mushrooms such as lingzhi (*Ganoderma lucidum*), shiitake (*Lentinus edodes*), and yiner (*Tremella fuciformis*) that have been collected, cultivated and used for hundreds of years, are being evaluated as edible and medicinal resources. Most traditional knowledge about the mushroom as a food and medicinal agent comes from these species. Traditionally, mushroom has been defined as a fleshy, aerial umbrella-shaped, fruiting body of macrofungi, and has been consumed by Asian people for over two thousand years because of the pleasant flavor and texture (Miles & Chang, 1997; Wasser, 1997). In the literature, it is widely accepted that ''mushroom'' is: a macro-fungus with a distinctive fruiting body that is large enough to be seen by the naked eye and to be picked up by hand (Chang & Miles, 1992).

The macrofungi with distinctive fruiting bodies commonly occurred in fungi of the class of *Basidiomycetes*, and sometimes in the class of *Ascomycetes*. Although truffle (genus tuber) and fruiting bodies of morel (both of them belong to the class *Ascomycetes*) have been reported for their artificial cultivation, no *Ascomycetes* mushroom has been successfully commercially cultivated (Hawksworth, 1998). Therefore, all the mushroom species mentioned in this article belong to the class of *Basidiomycetes*. The bioactivity of *Basidiomycetes* mushrooms was confirmed by Lucas for the first time in 1957 (Lucas, 1957). Lucas isolated a substance from *Boletus edulis* that has a significant inhibitory effect against Sarcoma S-180 tumor cells. Carrying out an extensive study in 1966, Gregory isolated the active substances from fruiting bodies of more than 200 *Basidiomycetes* mushroom species, and from 7000 culture media produced by applying submerge fermentation to the correspondent mushroom types (Gregory, 1966). The antitumor assays of these active substances were applied to three rodent animal models and revealed that the polysaccharides isolated from 22 mushroom species and 50 culture media displayed an inhibitory effect on tumor cells, including Sarcoma S-180, adenocarcinoma 755, and leukemia L-1210. Since then, scientists have subsequently isolated antitumor mushroom polysaccharides from *G. lucidum* (Miyazaki & Nishijima, 1981; Mizuno, 1997), *Poria cocos* (Kanayma, Togami, Adachi, Fukai, & Okumoto, 1986), *L. edodes* (Chihara, 1969; Chihara, Hamuro, Maeda, Arai, & Fukuoka, 1970; Hobbs, 2000), *Coriolas versicolor* (Hiroshi & Takeda, 1993), *Grifola*

frondosa (Cun *et al*., 1994; Mizuno, Ohsawa, Hagiwara, & Kuboyama, 1986; Mizuno & Zhuang, 1995) and *Auricularia auricular-judae* (Ukai *et al*., 1982, 1983). Table 1 lists the source, type and bioactivities of some distinctive fungal polysaccharides with their demonstrated activities. Bioactive polysaccharides can be isolated from mycelium, the fruiting body, and sclerotium, which represent three different forms of a macrofungi in the life cycle. The 28 species listed in Table 1 have been extensively studied in the past thirty years. Among them, several polysaccharides and polysaccharide conjugates have been commercialized for the clinical treatment of patients undergoing anticancer therapy. They are schizophyllan, lentinan, grifolan, krestin (polysaccharide–peptide complex) and PSK (polysaccharide–protein complex).

The natural antitumor polysaccharides isolated from mushroom include acidic and neutral ones with different types of glycosidic linkages, while some are bound to protein or peptide residues such as polysaccharide–protein or –peptide complexes (Cun *et al*., 1994; Jong, Birmingham, & Pai, 1991; Mizuno & Zhuang, 1995). In addition to the primary structure, a higher structure of polysaccharides, such as chain conformation, also plays an important role in their antitumor activities (Wasser, 2002). Most polysaccharides have remained classified as nonspecific bioactive substances because their exact mode of action was unknown, and chain conformation of their active components was undefined.

This article reviews recent work in this field with emphasis on the structure-bioactivity relationship, the extraction process, structural features, physical properties, antitumor activities, and cellular mechanism.

Extraction and purification procedures

Mushroom polysaccharides exist as a structural component of fungal cell wall. Fungal cell wall is composed of two major types of polysaccharides: one is a rigid fibrillar of chitin (or cellulose), the other one is a matrix-like β-glucan, α-glucan and glycoproteins (Ruiz-Herrera, 1956). Schizophyllan, a water-soluble $1\rightarrow6$ branched β-$(1\rightarrow3)$-glucan loosely attached to the outer layer of the cell wall, is secreted to the extracellular matrix (exopolysaccharides or extracellular polysaccharides). These water-soluble glucans fill the outer layer resisting the external pressure. The inner-layer polysaccharide of schizophyllan is alkali-insoluble and remains insoluble even in 40% KOH at 100 °C. It has been shown that the inner-layer polysaccharide of the schizophyllum commune is a glucan–chitin complex because the chitinase-treated polysaccharide was found to be a water-soluble glucan (Sietsman & Wessels, 1979; Wessels & Sietsman, 1979).

Selection of an extraction method depends on the cell wall structure. Hot water extraction has been a popular approach. Mizuno (Mizuno, 1996) developed reliable procedures for successful extraction of polysaccharides from fruiting bodies or cultured mycelia. In general, the extraction method involves elimination of low molecular substances from mushroom material with 80% ethanol, followed by three successive extractions with water (100 °C, 3 h), 2% ammonium oxalate (100 °C, 6 h), and 5% sodium hydroxide (80 °C, 6 h). The extraction with hot water yielded water-soluble polysaccharides, and the extraction with alkali solution yielded the water-insoluble ones. The extraction method can be varied based on the structure and water-solubility of polysaccharides, but the basic rule is to break the cell wall from outer layer to the inner layer with mild-to-strong extraction conditions (pH and temperature).

Extracted polysaccharides can be further purified using a combination of techniques, such as ethanol precipitation, fractional precipitation, acidic precipitation with acetic acid, ion-exchange chromatography, gel filtration, and affinity chromatography. The ethanol precipitation excludes the impurities from the polysaccharides. The separation of acidic and neutral polysaccharides can be achieved by anion-exchange chromatography on a DEAE-cellulose column. The neutral polysaccharide in the mixture is first eluted by an appropriate running buffer; the acidic polysaccharide is then eluted at a higher salt concentration. Neutral polysaccharides can be further separated into α-glucans (adsorbed fraction) and β-glucans (non-adsorbed fraction) with the help of gel filtration and affinity chromatography. Affinity chromatography is a process of bioselective adsorption and subsequent recovery of a compound from an immobilized ligand. This process now allows for the highly specific and efficient purification of some carbohydrates. The Sigma-Aldrich Co. has developed several carbohydrate-binding matrices which have high specific affinity for many diverse glycoproteins and carbohydrates (http://www.sigmaaldrich.com/Area_of_Interest/Life_Science/Proteomics_and_Protein_Expr_/Protein_Analysis/Chromatography/Affinity_Chromatography.html). L2507 and L5147 are used for the purification of both O-linked glycoproteins, and those glycoproteins containing α-D-galactose. Specificity of L8775 is directed to the nonreducing end of the terminal α-D-mannosyl residue of glycoconjugates. L4018 has an affinity for terminal α-D-mannosyl and α-D-glucosyl residues, and is used for the separation of α- and β-glucans in combination with gel filtration chromatography. The polysaccharide with a broad polydispersity can be fractionated by stepwise precipitation or preparative gel permeation chromatography, yielding polysaccharides with different molecular weights and low polydispersity. It should be noted that the particular fractionation procedure scheme in each case depends on the polysaccharide composition of the original material, involving their molecular weight, branching degree and pattern of branches.

Structural and physical properties

Structural features and analytical techniques

Polysaccharides with strong antitumor action differ greatly in their chemical structures. Antitumor activity is exhibited by a wide range of glycans extending from

Table 1. Source, type and bioactivity of some macrofungal polysaccharides

Fungi source	References	Polysaccharide source	Type	Main bioactivity
Pleurotus tuber-regium	Zhang, Cheung, & Zhang, 2001; Zhang, Chiu, Cheung, & Ooi, 2006; Zhang, Zhang, & Cheung, 2003	Sclerotium, mycelium	β-D-glucan	Hepato-protective, anti-breast cancer
Ganoderma lucidum	Miyazaki & Nishijima, 1981; Mizuno, 1997	Fruiting body, culture broth	Heteroglycan, mannoglucan, glycopeptide	Hyperglycemia, immunomodulating, antitumor, antioxidative, anti-decrepitude
Auricularia auricula	Ukai *et al.*, 1983; Ukai *et al.*, 1982	Fruiting body	Glucan	Hyperglycemia, immunomodulating, antitumor, antiflammatory, antiradiative
Schizophyllum commune	Yamamoto, 1981	Mycelium	Glucan, schizophyllan[a]	Antitumor
Hericum erinaceus	Kawagishi, Ando, & Mizuno, 1990; Mizuno, 1992; Mizuno, 1998	Fruiting body, mycelium	Heteroglycan, hetero-glycanpeptide	Hyperglycemia, immunomodulating, antitumor
Lentinus edodes	Chihara, 1969; Chihara *et al.*, 1970; Hobbs, 2000	Culture broth, fruiting body	Mannoglucan, polysaccharide–protein complex, glucan, lentinan[a]	Immunomodulating, antitumor, antiviral
Sclerotinia sclerotiorum	Palleschi, Bocchinfuso, Coviello, & Alhaique, 2005	Sclerotium	Glucan, scleroglucan (SSG)[a]	Antitumor
Polystictus versicolar	Cui & Chisti, 2003	Fruiting body, culture broth, mycelium	Heteroglycan, glycopeptide, krestin (PSK)[a]	Immunomodulating, antitumor, antiradiative, hyperglycemia, antiflammatory
Grifola frondosa	Cun *et al.*, 1994; Zhuang *et al.*, 1994; Zhuang, Mizuno, Ito, Shimura, & Sumiya, 1993	Fruiting body	Proteoglycan, glucan, galatomannan, heteroglycan, grifolan[a]	Immunomodulating, antitumor, antiviral, hepatoprotective
Inonotus obliquus	Kim *et al.*, 2005	Fruiting body, mycelium	Glucan	Antitumor, immunomodulating
Agaricus blazei	Mizuno, 1992; Mizuno, 1998	Fruiting body, mycelium	Glucan, heteroglycan, glucan protein, Glucomannan–protein complex	Antitumor
Flammulina velutipes	Zeng, 1990	Fruiting body, mycelium	Glucan–protein complex, glycoprotein	Antitumor, antiflammatory, antiviral, immunomodulating
Ganoderma applanatum	Nakashima, Umeda, & Kanada, 1979	Fruiting body	Glucan	Antitumor
Polypours umbellatus	Yang *et al.*, 2004	Mycelium	Glucan	Antitumor, immunomodulating
Clitopilus caespitosus	Liang, Miao, & Zhang, 1996	Fruiting body	Glucan	Antitumor
Pleurotus citrinopileatus	Wang, Hu, Liang, & Yeh, 2005	Fruiting body	Galactomannan	Antitumor
Trametes robiniophila	Zhang, 1995	Mycelium	Proteoglycan	Immunomodulating, hepatoprotective, anticancer
Tremella fuciformis	Huang, 1982	Fruiting body, mycelium, culture broth	Heteroglycan	Hyperlipidemia, hyperglycemia, immunomodulating, antitumor, anti-decrepitude, anti-thrombus
Tremella aurantialba	Liu, Xie, Su, Han, & Liu, 2003	Fruiting body, mycelium	Heteroglycan	Immunomodulating, hyperglycemia
Pleurotus ostreatus	Solomko, 1992	Fruiting body	Glycoprotein	Antitumor, hyperglycemia, antioxidant
Morchella esculenta	Duncan *et al.*, 2002	Fruiting body	Heteroglycan	Hyperglycemia, antitumor
Omphalia lapidescens	Saito, Nishijima, Ohno, Yadomae, & Miyazaki, 1992	Fruiting body	Glucan	Antiflammatory, immunomodulating
Phellinus linteus	Kim, Choi, Lee, & Park, 2004	Fruiting body	Glucan	Antitumor
Armillariella tabescens	Kiho, Shiose, Nagai, & Ukai, 1992	Mycelium	Heteroglycan	Antitumor
Dictyophora indusiata	Hara *et al.*, 1991	Fruiting body	Heteroglycan, mannan, glucan	Antitumor, hyperlipidemia

Table 1. (*continued*)

Fungi source	References	Polysaccharide source	Type	Main bioactivity
Peziza vericulosa	Mimura, Ohno, Suzuki, & Yadomae, 1985	Fruiting body	Proteoglycan, glucan	Immunomodulating, antitumor
Tricholoma mongolium	Wang, Ooi, Ng, Chiu, & Chang, 1996	Fruiting body	Glucan	Antitumor
Cordyceps sp	Hsu, Shiao, Hsieh, & Chang, 2002	Fruiting body, mycelium, culture broth	Glucan, heteroglycan	Antitumor, immunomodulating, antitumor, heperglycemia

[a] Commercially developed polysaccharide products.

homopolymers to highly complex heteropolymers (Ooi & Liu, 1999). A wide range of antitumor or immuno-stimulating polysaccharides of different chemical structures from higher *Basidiomycetes* mushrooms have been investigated, and the main types are listed in Table 2. Monosaccharide types of these antitumor polysaccharides contain glucose, galactose, mannose, xylose, arabinose, fucose, ribose and glucuronic acid. In some mushroom species, polysaccharides are bound with proteins or peptides as a polysaccharide–protein or –peptide complex which showed higher potent antitumor activity (Cui & Chisti, 2003). In addition to the well-known antitumor 1→3-β-glucans, a wide range of biologically active glucans with other structures are described in Table 2. These glucans are linear or branched molecules having a backbone composed of α- or β-linked glucose units, and some of them contain side chains that are attached at different positions. Heteroglucan side chains contain glucuronic acid, xylose, galactose, mannose, arabinose, or ribose, and may have different combinations. Another large group of bioactive polysaccharides is called heteroglycans which are classified as galactans, fucans, xylans, and mannans by individual sugar components in the backbone. Heteroglycan side chains may contain arabinose, mannose, fucose, galactose, xylose, glucuronic acid, and a glucose moiety as a main component, or in various combinations.

The structure of naturally occurring glycans is so diversified that it is difficult to define a universal protocol for their analysis. The building blocks (derived from sugar residues) of complex polysaccharides exhibit very similar structures, but their differentiation (diversified linkage style) is more inclusive than that of amino acids. The primary structure of a polysaccharide is defined by monosaccharide composition, configuration of glycosidic linkages, position of glycosidic linkages, sequence of monosaccharides, as well as the nature, number and location of appended non-carbohydrate groups. The analytical methods used to determine primary structures of polysaccharides are described in Table 3 (Varki *et al.*, 1999). Monosaccharide analysis provides precise molar ratios of individual sugars, and may suggest the presence of specific oligosaccharide classes such as N- or O-glycans. Monosaccharide composition analysis involves cleavage of all glycosidic linkages, fractionation of the resulting monosaccharides, and detection and quantification of each monosaccharide. Several methods for this purpose are listed in Table 3. In the early 1990s, high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was developed to supplement traditional methods because this process does not require monosaccharide derivatization. In the mid 1990s, fluorescent derivatives of monosaccharides were produced using reductive amination. This technique became popular in conjunction with the application of reverse-phase HPLC with on-line fluorescent detection, or gel eletrophoresis or high-performance capillary electrophoresis. Tagging polysaccharides with a fluorescent compound has resulted in an increase in detection sensitivity that extends into the fentomole range.

The positions of glycosidic linkages can be analyzed by enzyme digestion, methylation analysis and NMR spectroscopy. The exoglycosidic digestion method is limited to a few enzymes of high specificity. In methylation analysis, polysaccharides are first converted to partially methylated acetyl alditol and then analyzed. Resulting chromatographic peaks are identified by a combination of their retention times and their electron impact–mass spectrometry (EI-MS) fragmentation patterns. This type of analysis indicates which residues are terminal and how each monosaccharide is substituted, as well as the occurrence of branching points. Methylation analysis also allows the determination of the ring size for each monosaccharide. However, methylation analysis does not provide information on the sequence of constituent residues and the type of anomeric configurations (i.e. α or β).

The anomerity (α or β) of each sugar residue can be determined by NMR spectroscopy. By now there are quite a few review papers and books concerning structural analysis of complex carbohydrates (Cui, 2005; Vliegelhart & Dorland, 1983). If a sufficient quantity of polysaccharides (5–20 mg) is available, the anomericity of a particular monosaccharide residue is more reliably determined by ^{1}H NMR spectroscopy. The anomeric resonances appear in a clear region in the spectrum and show characteristic doublets with a splitting that is significantly larger for β anomers than for α anomers. NMR spectroscopy is the only method that has the potential for full structural characterization of a polysaccharide, with little or no assistance from other methods. Complete structural elucidation requires the full assignment of both the ^{1}H and ^{13}C NMR spectra of the oligosaccharide. Full assignment of ^{1}H and

Table 2. Chemical structures of antitumor mushroom polysaccharides

Polysaccharide	Linkages and types	Mushroom resources	Main chain	Branch	M_w
Homoglucans	(1→3)-β-D-glucan with 1–6 branches	*Lentinan from Lentinus edodes* (Mizuno, 1997)	(1→3)-β-D-glucan	(1→6)-β-	5×10^5
		Schizophyllan from Schizophyllum commune (Yamamoto, 1981)	(1→3)-β-D-glucan	(1→6)-β-	
		Grifola from Grifola frondosa (Zhuang *et al.*, 1994)	(1→3)-β-D-glucan	(1→6)-β-	5×10^5
		Scleroglucan from Sclerotium sclerotia (Palleschi *et al.*, 2005)	(1→3)-β-D-glucan	(1→6)-β-	
		An alkali-soluble glucan from Pleurotus tuber-regium (Zhang *et al.*, 2003)	(1→3)-β-D-glucan	(1→6)-β-	2×10^5
	Linear (1→3)-β-D-glucan	*Auricularia auricula* (Ukai *et al.*, 1983) *Lyophyllum decastes* (Ukawa, Ito, & Hisamatsu, 2000)	(1→3)-β-D-glucan	—	—
	Linear (1→6)-β-glucan	*Armillariella tabescents* (Kiho *et al.*, 1992)	(1→6)-β-D-glucan	—	—
	(1→3)-β-D-glucan with 1–2 or 1–6 branches	*Pachyman from Poria cocos* (Kanayma *et al.*, 1986)	(1→3)-β-D-glucan	(1→2)-β- or (1→6)-β-	1×10^5
	(1→3)-α-glucan	*Armillariella tabescens* (Ukawa *et al.*, 2000) *Linear α-(1-3)-glucan from Amanita muscaria* (Kiho *et al.*, 1992)	(1→3)-α-glucan	—	—
	(1→4)-α-; (1→6)-α-glucan		(1→6)-α-glucan	(1→4)-α-	—
	(1→4)-α-; (1→6)-β-glucan	*Agricus blazei* (Mizuno, 1992; Mizuno, 1998)	(1→6)-β-D-glucan	(1→4)-α-	—
	(1→6)-β-; (1→3)-α-glucan		(1→3)-α-glucan	(1→6)-β-	—
Heteroglucans	(1→3)-β-glucuronoglucan	*Ganoderma lucidum* (Mizuno, 1998)	(1→3)-β-glucuronoglucan	Glucuronic acid	5.3×10^4
	Xyloglucan	*Grifola frondosa* (Mizuno & Zhuang, 1995; Zhuang *et al.*, 1994)	Glucan	Xylose	—
		Polyporus confluens (Sugiyama *et al.*, 1992)			—
		Pleurotus pulmonarius (Wasser, 2002)			—
	Arabinoglucan	*Ganoderma tsugae* (Wang *et al.*, 1993)	Glucan	Arabinose	—
	Riboglucan	*Agricus blazei* (Mizuno, 1992; Mizuno, 1998) *Flammulina velutipes* (Zeng, 1990)	Glucan	Ribose	—
	Galactomannoglucan	*Hohenbuehelia serotina* (Ma, Mizuno, & Ito, 1991) *Leucopaxillus giganteus* (Wasser, 2002)	Glucan	Galactose, and mannose	—
	Galactoxyloglucan	*Hericium erinaceus* (Kawagishi, Kanao *et al.*, 1990; Mizuno, 1992; Mizuno, 1998)	Glucan	Galactose and xylose	—
	Mannoxyloglucan	*Grifolan frondosa* (Cun *et al.*, 1994; Mizuno & Zhuang, 1995; Zhuang *et al.*, 1994)	Glucan	Mannose and xylose	—
	Xylogalactoglucan	*Inonotus obliquus* (Kim *et al.*, 2005)	Glucan	Xylose, galactose	—

Table 2. (*continued*)

Polysaccharide	Linkages and types	Mushroom resources	Main chain	Branch	M_w
Heterogalactan	Glucogalactan	*Ganoderma teugae* (Peng, Zhang, Zeng, & Kennedy, 2005)	Galactan	Glucose	—
	Arabinogalactan	*Pleurotus citrinopileatus* (Wang *et al.*, 2005)	Galactan	Arabinose	—
	Fucogalactan	*Sarcodon aspratus* (Mizuno *et al.*, 2000)	Galactan	Fucose	—
	Mannogalactan	*Pleurotus pulmonarius* (Wasser, 2002)	Galactan	Mannose	—
	Fucomannogalactan	*Grifola frondosa* (Cun *et al.*, 1994; Mizuno & Zhuang, 1995; Zhuang *et al.*, 1994)	Galactose	Fucose mannose	—
Other heteroglycans	Xylan	*Hericium erinaceus* (Kawagishi, Kanao *et al.*, 1990; Mizuno, 1992; Mizuno, 1998)	Xylan	—	—
	Glucoxylan		Xylan	Glucose	—
	Mannogalactofucan	*Grifola frondosa* (Cun *et al.*, 1994; Mizuno & Zhuang, 1995; Zhuang *et al.*, 1994)	Fucan	Mannose and galactose	—
	Mannoglucoxylan	*Hericium erinaceus* (Kawagishi, Kanao *et al.*, 1990; Mizuno, 1992; Mizuno, 1998)	Xylose	Mannose, glucose	—
	(1→3)-α-mannan	*Dictyophora indusiata* (Hara *et al.*, 1991)	(1→3)-α-mannan	—	—
	Glucomannan		Mannan	Glucose	—
	(1→2)-β-; (1→3)-β-glucomannan	*Agricus blazei* (Kawagishi, Kanao *et al.*, 1990; Mizuno, 1992; Mizuno, 1998)	(1→3)-β-linked mannose	(1→2)-β-glucan	—
	Galactoglucomannan		Mannan	Galactose and glucose	—
Polysaccharide–protein/peptide complexes	Polysaccharide–peptide complex	*Coriolus versicolor* (Cui & Chisti, 2003)	α-1,4 and β-1,3 glucoside linkage; containing arabinose, rhmanose, but no fucose	Peptide mainly consists of aspartic and glutamic acids	1×10^5
	Polysaccharide–protein complex		α-1,4 and β-1,3 glucoside linkage; containing fucose, but no arabinose and rhmanose	Peptide mainly consists of aspartic and glutamic acids	1×10^5

^{13}C NMR spectra can be accomplished by using a combination of two-dimensional (2D) NMR techniques consisting of correlated spectroscopy (COSY) and total correlated spectroscopy (TOCSY) for ^{1}H, and then heteronuclear single-quantum coherence (HSQC) for ^{13}C. 2D heteronuclear multi-bond correlation (HMBC) is the key experiment for sequence determination. However, HMBC is not a very sensitive technique so that a relatively large amount of sample is needed for the 2D ^{1}H^{13}C NMR experiments. In the cases where there is not enough sample for this experiment, polysaccharide sequencing relies exclusively on 2D ^{1}H NMR spectroscopy, using through-space effects (nuclear overhauser effect, NOEs) as a source of evidence for linking positions and sequence.

The structure characterization of glycans in a typical glycoprotein has been investigated in recent years (Verbert, 1995). A polysaccharide–protein complex presents one or more diffuse bands when it is separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then electroblotted onto polyvinylidene difluoride (PVDF) membranes; this results from heterogeneity in the carbohydrate moiety. Some polysaccharide–proteins or –peptides of very high molecular weight do not enter ordinary gels, or they migrate as smears. In such a situation, agarose gels or combination of polyacrylamide–agarose gels can be used. Visualized by protein staining reagents (recently many analytical options to verify a positive carbohydrate staining reaction are available depending on the type and

Table 3. Common methods for primary structure analysis (modified from reference (Varki *et al.*, 1999, chap. 38))

Primary structure features	Methods	Derivatization	Information obtained
Monosaccharide composition (nature and number of monosaccharide, including absolute configuration of D or L, and ring size of pyranose or furanose)	GLC–FID Developed in early 1960s	Complete derivatization to produce volatile compounds; complete derivatization with chiral aglycone	Type, quantity and D or L configuration of monosaccharide
	GLC–MS Developed in 1970s	Complete derivatization to produce volatile compounds	Type and quantity of monosaccharides
	HPLC Developed in 1980s	Pre- and post-column derivatization: fluoresecent tagging of reducing end	Type and quantity of monosaccharides
	HPAEC-PAD Developed in early 1990s	Not needed	Type and quantity of monosaccharides
Configuration (α, β) of anomeric carbon	IR	Not needed	Configuration of α or β
	NMR	Not needed	Configuration of α or β
Positions of glycosidic linkages	Exoglycosidase digestion with specific enzymes (limited to a few enzymes of high specificity) with proper on-line detectors such as HPLC, HPCE	Fluorescent tag may need to be introduced at reducing end	Presence of residues in α or β linkages to specific positions of the underlying saccharides
	MS	Complete derivatization to produce volatile compounds	Linkage type and position might be inferred from specific fragmentations across sugar rings
	NMR	Not needed	Anomericity of each monosaccharide residue obtained from the chemical shift and coupling constant of H-1 linkage positions deduced from 2-D HMBC experiment
Sequence	NMR	Not necessary	Sequence may be inferred by comparison with standards; sequence deduced from 2-D HMBC experiment

quantity of sample), an appropriate piece of PVDF membrane is subjected to sequential hydrolysis with mild acid (0.2M trifluoroacetic acid, TFA), 2M TFA and finally 6M HCl at 100 °C for 24 h to release acidic sugars, neutral sugars and amino acids, respectively. Complete removal of N- and O-glycans can also be achieved by using enzymes and chemical treatments such as hydrazinolysis, β-elimination, or hydrogen fluoride treatment (Ausubel *et al.*, 1996; Jackson & Gallagher, 1997; Townsend & Hotchkiss, 1997). The high sensitivity of current instrumentation facilitates on-line composition analysis of sequential hydrolysates released by treating polysaccharide–protein complexes what are blotted onto polyvinylidene difluoride (PVDF) membranes with acids (Zdebska & Koscielak, 1999). This method has the benefit of assuring that the hydrolytes are easily recovered, and leaving any peptide or protein bound on the membrane.

The extent to which the primary structure of a polysaccharide has been assessed depends on the available techniques. The contribution from a variety of disciplines will be required to advance such technologies. To put together all the pieces of information for the primary structure will be an interesting challenge for the carbohydrate chemists.

Conformational properties and analytical methods

The conformational aspects of a polysaccharide include conformation of each monosaccharide, orientation of monosaccharides with respect to each other, and flexibility of the spatial structure which is defined by dihedral angles, torsion angles around glycosidic bonds, inter-atomic distances and dynamic parameters, respectively. For most mushroom polysaccharides, the secondary- and higher-order structures in solution are not readily defined, due to their inherent flexibility. Characterization of polysaccharide dynamics by both experimental and theoretical systems remains an area of active research. With the development of high resolution instrumental processes, such as various light scattering techniques (i.e. light scattering, x-ray and neutron scattering), x-ray diffraction analysis, small-angle neutron scattering (SANS), atomic force microscopy (AFM) and high resolution NMR, it is possible to study the conformation and 3D structure of a polysaccharide at the molecular level. By using molecular mechanics and computer-assisted energy minimization methods, it is possible to simulate and visualize the 3D structure of polysaccharides.

Conformation and chain rigidity

Based on the molecular parameters obtained from laser light scatterings (LLS) and SANS, Rees and his coworkers (Rees, 1969; Rees & Scott, 1969, 1971) simulated linear and branched pyranosic glucans and delineated their most probable conformations. Fig. 1 summarizes the conformations of glucan with various glycosidic linkages. 1→4-β-glucan and 1→3-α-glucan have the similar extended and

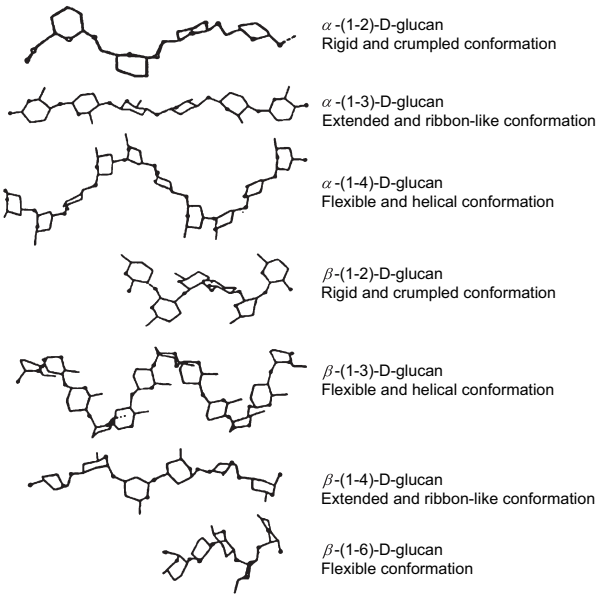

Fig. 1. Regular α- and β-glucan conformations, deduced from computer outputs. Modified from Rees and coworkers (Rees & Scott, 1971).

ribbon-like conformation; 1→3 β-glucan and 1→4 α-glucan have the flexible and helical conformation; 1→2 β-glucan and 1→2 α-glucan have rigid and crumpled conformation. 1→6 β- and 1→6 α-glucans are quite different from glucans in the other linkages because they have many possible conformations. This is because residues connected through the 1→6 linkage are separated by three bonds rather than two, so that its freedom of rotation is much higher than that of other linkages. Therefore, the outstanding property of 1→6 linkage is its flexibility. Most of Rees' (Rees, 1969; Rees & Scott, 1969, 1971) predictions about the conformation of glucan have been attested by experimental analysis, except for those of (1→3)-α, (1→2)-α, (1→2)-β, (1→6)-β- and (1→6)-α-glucan. This is mainly due to the fact that the first three have poor packing ability in organic cells and their native forms seldom occur in nature; (1→6)-β- and (1→6)-α-glucan show typical flexible chain characteristics which prevent further analysis. Burton & Brant (Burton & Brant, 1983) introduced an energy function for conformational analysis of glucans with various linkages. They calculated molecular parameters including characteristic ratio (C_∞), persistence length (q) and configuration entropy (ΔS_c) to describe chain rigidity. The molecular parameters of some glucans are listed in Table 4 for quantitative comparison of their extension and flexibility. According to the q value (a larger q stands for higher chain rigidity), the order of chain rigidity of glucans is as follows: (1→4)-β- > (1→3)-α- > (1→4)-α- > (1→3)-β- > (1→6)-α- > (1→6)-β-. Such a conclusion is consistent with that of Rees (Rees, 1969).

Helical conformation

The helical conformation is an important structure that was found in antitumor mushroom polysaccharides. Six mushroom polysaccharides that have been used for treatment of certain cancers adopt triple helical conformation in solution. They are schizophyllan (Kashiwagi, Norisuye, & Fujita, 1981; Sato, Norisuye, & Fujita, 1983; Yanaki, Norisuye, & Fujita, 1980), scleroglucan (Sato *et al*., 1983), lentinan (Saito, Yoshioka, Yakoi, & Yamada, 1990), curdlan (Saito *et al*., 1990), cinerean (Gawronski, Aguirre, Conrad, Springer, & Stahmann, 1996; Gawronski, Conrad, Springer, & Stahmann, 1996; Gawronski *et al*., 1997) and (1→3)-β-D-xylan (Itou, Teramoto, Matsuo, & Suga, 1986). A linear water-soluble (1→3)-β-D-glucan isolated from *Auricularia auricula* that existed as a single helical chain in solution also showed very strong antitumor activity (Zhang & Yang, 1995). Since helical conformation has been considered a very important factor by playing a significant role in the biological recognition within cells, a basic understanding of the conformation and conformation transition is essential for medicinal applications. It has been shown that schizophyllan, which has the primary structure of (1→3)-β-D-glucan with one 1→6 branches for every three β-(1→3)-glucopyranosides, adopts a triple helical conformation in water with the molecular parameters of 2170 nm^{-1} for molecular weight per contour length (M_L), 150 nm for persistence length (q) and 0.30 nm for distance per turn of helix (h) (Kashiwagi *et al*., 1981). It was found that schizophyllan not only adopts a triple helical conformation in water, but also has a random coil conformation in dimethylsulfoxide (DMSO) (Sato *et al*., 1983). When water was added to DMSO, the single chain of schizophyllan associated because of the formation of hydrogen bonds. McIntire and Brant (McIntire & Brant, 1999), and Young and Dong (Young & Dong, 2000) have

Table 4. Quantitative comparison of extension and flexibility of several glucans

Polysaccharides	C_∞	q	ΔS_c	Description of conformation
α-(1-3)-D-glucan	32	70	0.15	Extended conformation, no pseudohelicity
β-(1-3)-D-glucan	3.1	15	1.1	Pseudohelical trajectory
α-(1-4)-D-glucan	5.0	28	−1.1	Pseudohelical trajectory, moderately compact chain
β-(1-4)-D-glucan	100	290	0.30	Extended conformation
α-(1-6)-D-glucan	1.5	7	4.0	Great randomness and tortuosity, frequent and random change in direction
β-(1-6)-D-glucan	1.7	6.9	3.4	Great randomness and tortuosity, frequent and random change in direction

Characteristic ratio (C_∞), persistance length (q) and configuration entropy (ΔS_c, cal/Kmol residue).

independently shown that the triple helical structure could be retrieved when the renaturation process was carried out in considerably dilute solutions. Such dissociation of triple helices and their reorganization has inspired researchers to study the interaction between a triple helical polysaccharide and DNA/RNA (Anada *et al*., 2004; Koumoto, Mizu, Sakurai, Kunitake, & Shinkai, 2004; Yang *et al*., 2005; Mizu, Koumoto, Kimura, Sakurai, & Shinkai, 2004). Sakurai dissociated schizophyllan into single chains by adding DMSO into the aqueous solution to break down the inter-molecular hydrogen bonds (Bae *et al*., 2004). The single chains were then added to an aqueous solution containing polynucleotides (poly(C)) for renaturation. It was found that two single chains could include one poly(C) chain to form a new triple helical complex. The complexation proceeds in a highly stoichiometric manner so that two schizophyllan repeating units and three poly(C) units are bound in the complex (Sakurai, Mizu, & Shinkai, 2001). Results from x-ray crystallographic studies indicated that the newly formed triple helical complex was quite similar in molecular parameters to the original polysaccharide, but showed higher rigidity so that a good AFM spectrum could be expected (Mizu, Koumoto, Kimura, Sakurai, & Shinkai, 2003). This interesting finding helps scientists to connect the biological function of triple helical polysaccharides to their interaction with DNA and RNA. Some Japanese researchers have found that the single chain of schizophyllan forms a macromolecular complex with poly(C), poly(A), poly(dA), or poly(dT), but doesn't form a complex with poly(G), poly(U), poly(I), poly(dG) and poly(dC) (Saenger, 1984, chap. 6). This nucleotide specificity presents evidence that the hydrogen bonds are essential to form the complex, because the former nucleotides have unoccupied hydrogen-bonding sites and the latter ones have used these hydrogen-bonding sites in the intramolecular aggregation (Numata *et al*., 2003; Saenger, 1984, chap. 6). These features imply that the single chain of schizophyllan can behave like a poly(G) in the double strand poly(C)/poly(G). This opens a door for new fields of application with polysaccharides in future gene technology, such as antisense DNA drugs, nonvirus vectors and affinity chromatography (Numata *et al*., 2003). X-ray diffraction analyses for another helical mushroom polysaccharide, lentinan (Bluhm & Sarko, 1977), have predicted five models including one single helix, two double helices, and two triple helices for the crystalline lentinan that is (1→3)-β-D-glucan having two 1→6 branches for every five (1→3)-β-glucopyranoside. Xu *et al*. (Xu, Zhang, Zhang, & Wu, 2004) indicated, by means of LLS and AFM (atomic force microscopy), that lentinan predominantly existed as triple helical chain in 0.5 M NaCl aqueous solution and as single flexible chain in DMSO. Zhang *et al*. (Zhang, Zhang, & Xu, 2005) also successfully observed the dynamic transition of triple helices−single helix-random coil of this glucan. Polysaccharides that take on triple helical conformation have clinical applications for the treatment of certain cancers such as human breast cancer (MCF-7), human promyelocytic leukemia (HL-60), and human liver cancer (HpG2). Elucidation of their conformation and three dimensional structures will be crucial for understanding their biological function.

Methods for conformation analysis

High resolution NMR is the method of choice to study the conformation and 3D structures of saccharides, with parameters represented by chemical shifts, coupling constants, nuclear overhauser effects (NOEs), and also relaxation time. Coupling constants can be used to evaluate the magnitude of the torsion angles, and NOE measurements (Xu & Allen, 1996) can provide estimations of distances between protons located in rather close proximity. Relaxation time measurements give information on the mobility and the behavior of molecules in solution.

The x-ray diffraction method is usually used to characterize the crystal structure of polysaccharides. Most polysaccharides are amorphous and poorly crystalline. Only a few helical polysaccharides with high rigidity showing ordered structure can form crystals for x-ray diffraction analysis. In most cases, x-ray diffraction measurements are conducted using orientated fibers or films prepared from concentrated polysaccharide solutions. During the fiber preparation, the molecules are forced to align approximately parallel along the helical axes. Although this organization is artificial, the information obtained may help to understand the ordered structures of polysaccharides that occur in solution. A typical x-ray diffraction analysis may provide information about the helical structures such as repeat spacing of the helix, helical screw symmetry, the unit cell dimensions and lattice type. However, the interpretation of x-ray data usually requires supplementation with molecular modeling analysis using existing stereochemical information derived from crystal structures of related mono- or oligosaccharides (Rao, Qasba, Balaji, & Chandrasekaran, 1998, chap. 2). Examples of x-ray diffraction structures are given for curdlan (Deslandes & Marchessault, 1980) and (1→3)-α-D-glucan (Ogawa, Okamura, & Sarko, 1981).

AFM has become an important tool during the past decades to examine the conformation of macromolecules (Ando *et al*., 2001). AFM employs particular atom probes to directly study the shape and conformation of biomacromolecules such as protein, DNA and polysaccharides under circumstances similar to a physiological environment. Much work in the microscopic observation of biomolecules has been dedicated towards observation of the double helix of DNA (Hansma, Laney, Bezanilla, Sinsheimer, & Hansma, 1995). Compared to protein and DNA, an AFM spectrum of a polysaccharide is difficult to achieve due to a more complicated structure, especially when branches and non-carbohydrate substituted groups occur in polymer chains. The difficulty is also caused by the fact that the radius of the tips of the probe is generally larger than the

pitch of the helix, and that the structure of the molecules is generally fairly flexible, and probably distorted by the imaging process. Successful observations are limited to a few triple helical polysaccharides because they have a very rigid structure that could be imaged by AFM (Camesano & Wilkinson, 2001; McIntire & Brant, 1998). McIntire (McIntire & Brant, 1998) and Stokke (Stokke, Elgsaeter, & Kitamura, 1994) and Zhang *et al.* (Zhang, Chen *et al.*, 2005) have independently observed three triple helical (1→3)-β-D-glucans (scleroglucan, schizophyllan, and lentinan, respectively) with AFM, and they have found that the natural glucans exhibited a rod-like architecture, which is expected from the studies on the dilute solution properties of those polymers. However, the resolution was not good enough to observe the helicity of these polysaccharides. Kunitake & Ohira (Kunitake & Ohira, 2002) also reported helical pattern from AFM observation on the schizophyllan molecule. Nevertheless, the observed helicity was not consistent with the crystallographic data. As mentioned previously, a single chain of schizophyllan forms a high rigid complex with DNA/RNA so that a good AFM spectrum could be expected. This finding provides a good method to directly observe the helical structure of polysaccharides using AFM. McIntire (McIntire & Brant, 1998) successfully observed the triple helical conformation of scleroglucan that possesses the primary structure of 1→6 branched (1→3)-β-D-glucan. They also observed triple helices−single helix-random coil transition when a treatment of heat was applied to this polysaccharide. Moreover, recent experiments (Piotr, Hongbin, Andres, Oberhauser, & Fernandez, 2002) have demonstrated that a force field can trigger conformational transitions in these molecules which cannot be observed by traditional NMR or x-ray crystallographic techniques. Therefore, AFM should be increasingly applied in analyzing the branching and the length of the branches in the polysaccharide, cross-linked network, as well as the dynamic procedure.

LLS and SAXS (small-angle x-ray scattering) have been widely used to characterize polymer chains in solution. The weight average molecular weight (M_w), the radius of gyration ($<s^2>^{1/2}$) and the second virial coefficient (A_2) can be determined (Johnson & Gabriel, 1994). The shape of the polymer molecule, whether it is spherical, random coiled, or rod like, can be analyzed from data of $<s^2>^{1/2}$ or $[\eta]$ as a function of molecular weight. These data can be analyzed in terms of Yamakawa's theories for unperturbed wormlike chains, in order to provide molecular parameters such as molecular weight per contour length (M_L), persistence length (q) and distance per turn of helix (h).

Small-angle neutron scattering (SANS) has become a preferred tool to analyze a variety of polymer systems, including pure and blend bulk polymers, phase-separated systems, miceller suspensions, and solutions, especially concentrated ones (Higgins & Benoit, 1997). Unlike light scattering, it is available only in a limited number of facilities around the globe. Labeling of polymers by deuterium, that is, straight synthesis of the polymer using deuterated compounds, is often required. SANS is therefore best suited where LLS fails, as for instance, with opaque systems such as miceller suspensions in which multiple light scattering complicates the scattering pattern. As with LLS, SANS provides information on the static structure of the system, but molecular length is smaller.

Computer programs have been developed to generate 3D structures of polysaccharides from their primary sequence (Tvaroska, 1989). Computer modeling has been used to analyze the conformational effects of steric interactions between atoms of the polymer skeletons for related polysaccharides. Over 99% of the conformations are thus excluded and virtually all of the remainders for each polysaccharide lie close together. In brief, the simulation includes four steps (Perez, Kouwijzer, Mazeau, & Engelsen, 1996). The first step starts with a description of the conformations of the monosaccharides and a thorough description of the conformation space that is available for a repeating unit. The results were used in the second step, aimed at generating a disordered polymer chain based on the assumption that due to the size and relative rigidity of the intervening monosaccharide units, the rotations at a particular linkage can be, under certain conditions, independent of the nearest neighboring interactions. In the third step, the appropriate modeling techniques will be used to construct the ordered state of polysaccharide strands. The generation of multiple helices can be then attempted in order to explore the occurrence of such multi-strand arrangements that may be energetically stable. The final step in the determination of the 3D structure of polysaccharides in the ordered states, is the investigation of the possible interactions among helices and interactions between helices and target molecules. Molecular simulation allows automatic searches for meaningful correlations between structures and functions, through exploratory data analysis. Structure-function or structure-property correlation could then be used to model and predict changes arising from structural alterations.

Antitumor activity and cellular mechanism

The involvement and importance of polysaccharides in tumor and cancer treatment were first recognized more than 100 years ago when it was found that certain polysaccharides could induce complete remission in patients with cancer (Nauts, Swift, & Coley, 1946). Ever since antitumor activity of macrofungal polysaccharides was first published by Chihara in the 1960s (Chihara, 1969), researchers have isolated structural diversified polysaccharides with strong antitumor activity. Unlike traditional antitumor drugs, these substances produce an antitumor effect by activating various immune responses in the host and cause no harm to the body (Wasser & Weis, 1999).

Mushroom polysaccharides have shown widely inhibitory effects towards many kinds of tumors including Sarcoma 180 solid cancers, Ehrlich solid cancer, Sarcoma

37, Yoshida sarcoma and Lewis lung carcinoma (Wasser & Weis, 1999). The proposed mechanism by which mushroom polysaccharides exert antitumor effect include: 1) the prevention of the oncogenesis by oral administration of polysaccharides isolated from medicinal mushrooms (**cancer-preventing activity**); 2) enhancement of immunity against the bearing tumors (**Immuno-enhancing activity**); and 3) direct antitumor activity to induce the apoptosis of tumor cells (**Direct tumor inhibition activity**).

The cancer-preventing activity of medicinal mushroom polysaccharides has been found with *Hypsizygus marmoreus* and its mechanism was due to the immunopotentiation of polysaccharides (Ikekawa, 2001). The cancer-preventive activity of medicinal mushroom polysaccharides has been observed in farmers whose main occupation was producing medicinal mushrooms such as *Flammulina velutipes* in Japan and *Agaricus blazei* in Brazil (Ikekawa, 2001). These farmers' cancer death rate was remarkably lower than that of the general population by 40%. Consequently, an animal study was conducted by feeding the control mice with an ordinary diet and the treated mice with medicinal mushroom polysaccharides from *F. velutipes* and *A. blazei* in a diet, before they were inoculated with tumors. At the end of the experiment, the number of mice that developed tumors on the test diet was compared with that in the control group. This decreased number of tumor bearing mice indicates the cancer-preventive activity of the polysaccharides (Ikekawa, 2001).

Mushroom polysaccharides exert their antitumor action mainly via activation of the immune response of the host organism (**Immuno-enhancing activity**). In other words, mushroom polysaccharides do not directly kill tumor cells. They help the host to adapt to various biological stresses and exert a nonspecific action on the host, supporting some or all of the major systems. Mushroom polysaccharides cause no harm and place no additional stress on the body, therefore, they are regarded as biological response modifiers. Immuno-enhancing activity has been found in many mushroom polysaccharides. Mushroom polysaccharides have been shown to produce over 50% reduction in tumor size and prolong the survival time of tumor bearing mice (Wasser, 2002). Lentinan produced over 90% reduction in tumor size, or complete regression in most of the tested animals. In addition, it showed prominent antitumor activity not only against allogenic tumors, but also against various synergic and autochthonous tumors (Wasser, 2002). Schizophyllan displayed antitumor activity against both the solid and ascite forms of Sarcoma 180, as well as against the solid form of Sarcoma 37, Erlich sarcoma, Yoshida sarcoma and Lewis lung carcinoma (Wasser, 2002). Most of the experimental immuno-enhancing activity testing was performed by inoculating the mice with tumor cells (Ooi & Liu, 1999). The polysaccharides were administered intraperitoneally to the mice for a short period of time, after tumors had developed. The reduction of the tumor size or the number of the complete regressive test animals, and the prolonged survival time of the tumor bearing mice, were evaluated as the inhibitory effect, indicating the immuno-enhancing activity of the mushroom polysaccharides. The host-mediated immunomodulating activity has been demonstrated by a number of studies. The antitumor effect of polysaccharides was lost in neonatal thymectomized mice (mice that have no thymus-dependent immune system), and decreased significantly by administration of anti-lymphocyte serum (Maeda & Chihara, 1971). The results suggest that the antitumor action of polysaccharides requires an intact T-cell component and that the activity is mediated through a thymus-dependent immune mechanism. The possible pathways of host-mediated actions from lentinan have been suggested (Chihara, 1992) and later modified by S. P. Wasser (Wasser & Weis, 1999). The pathway explained the possible immune mechanism of lentinan, showing that the administration of lentinan can promote potentiation of response of precursor T cells and macrophages to cytokines produced by certain groups of lymphocytes after specific recognition of tumor cells (Chihara, 1992). The induction of a marked increased in the amounts of TNF-α (tumor necrosis factor), 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). In addition, lentinan is able to restore the suppressed activity of helper T cells in the tumor bearing host to their normal state, leading to the complete restoration of humoral immune responses (Maeda, Watanabe, Chihara, & Rokutanda, 1988). Moreover, it has been reported that the delayed-type hypersensitivity response induced at tumor sites by lentinan, and the subsequent infiltration of immune effector cells, such as natural killer cells and cytotoxic T lymphocytes, is an important mechanism of antitumor action for lentinan (Suzuki, Iwashiro, Takatsuki, Kuribayashi, & Hamuro, 1994). In recent years, it has been proposed that lentinan inhibits hepatic metastasis in adenocarcinoma-26 bearing mice by activating Kupffer cells (Taki *et al.*, 1995). Therefore, it remains to be clarified which immunomodulatory effects induced by lentinan are critical for tumor rejection. Schizophyllan is similar to lentinan in composition and antitumor activity, as well as in their mechanism for antitumor action (Jong *et al.*, 1991). However, the kinetics of gene expression of cytokines in schizophyllan is different in peritoneal exudate cells, splenocytes, and liver cells (Nemoto, Ohno, Saito, Adachi, & Yasomae, 1993; Okazaki, Adachi, Ohno, & Yadomae, 1995). Grifolan, which is isolated from *Grifola frondosa* and similar to schizophyllan in primary structure, is a novel macrophage activator that enhances mRNA levels of IL-6, IL-1, and TNF-α macrophages (Adachi, Okazaki, Ohno, & Yadomae, 1994).

Direct tumor inhibition activity has been documented in many mushroom polysaccharides (Wang *et al.*, 2002). Although the anti-proliferative effect of polysaccharides towards tumor lines *in vitro* remains unclear, some studies

indicate that incubation of polysaccharides together with tumor cells could change the expression of signals within tumor cells. That could arrest the cell cycle and generate apoptosis, which explains the *in vitro* anti-proliferative effect of polysaccharides (Chen & Chang, 2004; Li, Kim, Kim, & Park, 2004; Lin *et al*., 2003). It has been reported that a polysaccharide–peptide complex (PSP) extracted from *Trametes versicolor* significantly reduced proliferation of MAD-MB-231 breast cancer cells (Chow, Lo, Loo, Hu, & Sham, 2003), as compared with the control. It was also found that a protein bound polysaccharide (PBP) isolated from *Phellinus linteus* had an anti-proliferative effect for SW480 human colon cancer cells (Li *et al*., 2004). In addition, polysaccharides isolated from *P. cocos*, *Lycium barbarum* and *Cladonia furcata* have been demonstrated to have novel anti-proliferative activities (Chen & Chang, 2004; Li *et al*., 2004; Lin *et al*., 2003; Zhang, Chen *et al*., 2005). These results suggest that mushroom polysaccharides not only stimulate the proliferation of T lymphocytes and the immune function through the immunopotentiation, which has been revealed in the last three decades (Zaidman, Yassin, Mahajna, & Wasser, 2005), but also have a direct action on the tumor cells. Nevertheless, little is known about the direct effect of polysaccharides on cancer cells. In recent years, some immunostaining techniques have shown that PSP increased the p21 expression and decreased the cyclin D1 expression (Chow *et al*., 2003). Molecular techniques have also been used to study the effect of PBP from *P. linteus*. It was found that the direct cytotoxicity of PBP was mediated by induction of apoptosis, by G2/M cell cycle arrest associated with a decrease in Bcl-2, by an increase in the release of cytochrome c, and by reduced expression of cyclin B1 (Li *et al*., 2004). Lycium Barbarum polysaccharide (LBP) treatment caused inhibition of human hepatoma QGY7703 cell growth with cell cycle arrest in S phase and apoptosis induction (Zhang, Chen *et al*., 2005). In our previous study of carboxymethylated polysaccharides (CMPTR) from *Pleurotus tuber-regium* it was found that CMPTR-treated MCF-7 cancer cells were associated with reduced expression of the cell cycle related proteins, cyclin D1 and cyclin E, and with decreased expression of Bcl-2 and increased expression of Bax. This suggests that CMPTR can directly inhibit the proliferation of MCF-7 by blocking the cell cycle and generating apoptosis. Recent studies on direct cytotoxicity of polysaccharides have also indicated that polysaccharides can directly inhibit the cancer cell proliferation in a dose- and time-dependent manner, which could be mediated through up-regulation of p21 and down-regulation of cyclin D1. They could also directly induce apoptosis in cancer cells which might be mediated by up-regulation of a pro-apoptosis Bax protein (Zaidman *et al*., 2005).

Structure-antitumor activity relationship

Structural features such as (1→3)-β- linkages in the main chain of the glucan and additional (1→6)-β- branch points, have been indicated as important factors in antitumor action. β-glucans containing mainly 1→6 linkages exhibit less activity, possibly due to their inherent flexibility of having too many possible conformations. However, antitumor polysaccharides may have other chemical structures, such as hetero-β-glucans (Mizuno, Saito, Nishitoba, & Kawagashi, 1995), heteroglycan (Gao, Seljelid, Chen, & Jiang, 1996), β-glucan–protein (Kawagishi, Kanao *et al*., 1990), α-manno–β-glucan (Mizuno *et al*., 1995), α-glucan–protein (Mizuno *et al*., 1995) and heteroglycan–protein complexes (Mizuno *et al*., 1996; Zhuang *et al*., 1993). It has been postulated that mushroom polysaccharides containing glucose and mannose may have some antitumor action because a polysaccharide receptor has been found on human macrophages, which has demonstrated high specificity for glucose and mannose (Lombard, 1994).

Triple helical conformation of (1→3)-β-glucans is regarded as an important structural feature for their immuno-stimulating activity. (1→3)-β-glucans exhibit a variety of biological and immuno-pharmacological activities related to their triple helical conformation. For example, when lentinan was denatured with DMSO, urea, or sodium hydroxide, its tertiary structure was lost while its primary structure was maintained; but its tumor inhibitory effect was lowered with progressive denaturation (Maeda *et al*., 1988). The same results were obtained when a correlation between antitumor activity and triple helical structure was investigated in schizophyllan (Yanaki, Ito, & Tabata, 1986). However, exactly how the triple helical conformation of (1→3)-β-glucan affects their antitumor action still remains unclear. Many of the biological and immuno-pharmacological activities such as macrophage nitrogen oxide synthesis and limulus factor G activation, are dependent on the triple helical conformation; while other activities, such as synthesis of interferon-γ and colony stimulating factor (Yadomae & Ohno, 2000), are independent from the triple helical conformation. It has been found that the (1→3)-β-glucan backbone structure is of more importance than the tertiary structure of the molecule, and that helps to explain why (1→3)-α-mannan, having similar backbone conformation with (1→3)-β-glucan, has shown comparable antitumor action to (1→3)-β-glucan.

Mizuno indicated that high molecular weight glucans appear to be more effective than those of low molecular weight. However, unlike (1→3)-β-glucans with medicinal properties that are strongly dependent on high molecular weight, ranging from 500 to 2000 kDa (Mizuno *et al*., 1996), medical properties of some mushroom polysaccharides like (1→3)-α-glucuronoxylomannans, are not strongly dependent on molecular weights. Their hydrolysate fractions containing glucuronoxylomannans with molelcular weights from 53 to 1000 Da are as effective as those fractions having higher molecular weights (Gao *et al*., 1996). It was also reported by Gao that differences in molecular weight had no obvious influence on the activity of the heteroglycans (Gao *et al*., 1996).

Future perspectives

Polysaccharides belong to a structurally diverse class of macromolecules, in which polymers of monosaccharide residues are joined to each other by glycosidic linkages. Compared with proteins and nuclear acids, polysaccharides offer the highest capacity for carrying biological information because they have the greatest potential for structural variability (Ohno, 2005). Such variability gives great flexibility in the precise regulatory mechanisms of various cell–cell interactions in higher organisms. Such variability also results in structural complexity, which has forced the study of this kind of biopolymer to have lagged far behind the other two major classes of biopolymers, proteins and nucleic acids (Varki *et al*., 1999, chap. 38). The future challenge is to define the 3D structure of polysaccharides and the structure–function relationship. This presents a good opportunity for scientists to elucidate the biological roles of polysaccharides and design high potential antitumor drugs based on the 3D structures.

Future challenge in this area lies in putting together all the elementary structural parameters determined by high resolution instrumental methods, and to construct the elementary clusters using computer-assisted methods. Such constructions will lead to the understanding of how the structural features, such as degrees of branches or rigidity, control the establishment of the unique type of antitumor activity. In turn, the control of such structural features, via the chemical routes or molecular biology techniques, will create a polysaccharide, for which a significant range of properties can be predicted.

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