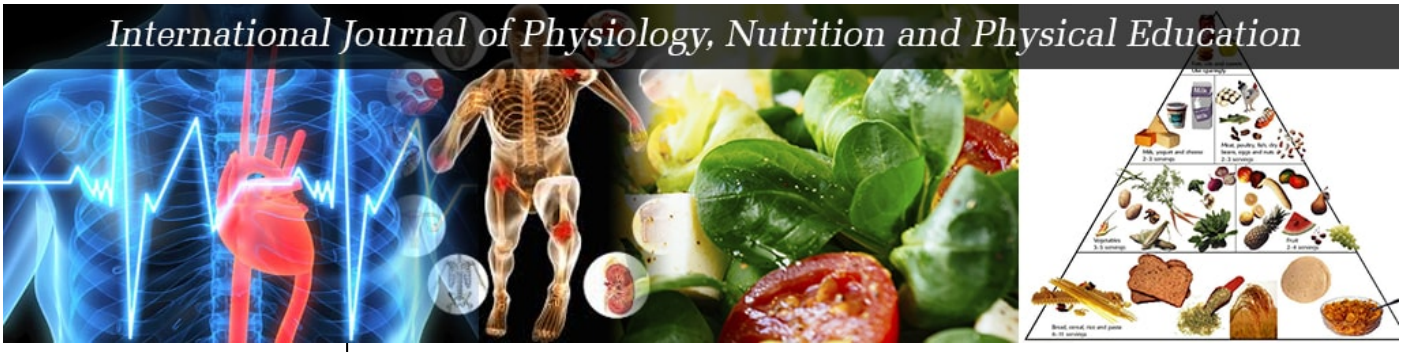

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Review on mushroom polysaccharides: Structural characterization and bioactivities

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Abstract
Mushrooms polysaccharides have attracted a great deal of attention due to the many healthy benefits they have demonstrated, such as immunomodulation, anticancer activity etc. Isolation of pure polysaccharides commonly involve several steps, and different techniques are actually available in order to increase extraction yield and purity. Several studies have demonstrated that the molecular structure and arrangement of monosaccharides significantly influence the biological activity; therefore, there is a broad range of methodical techniques for the elucidation of chemical structures. Different polysaccharides have been isolated from mushrooms, most of them consisting of β-linked glucans, such as lentinan, pleuran, schizophyllan, calocyban, and ganoderan. This article reviews the most important methods of polysaccharide extraction and structural characterization, and some of the most important polysaccharides extracted from mushrooms and the healthy benefits they provide.

Keywords: Mushroom, polysaccharide, structure, NMR, bioactivity

1. Introduction
For millennia, mushrooms have been valued by human kind as an edible and medical resource. A number of bioactive molecules, including 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 [1-8]. Many Basidiomycetes mushrooms contain biologically active polysaccharides in fruit bodies, cultured mycelium, and culture broth. Dochez and Avery made the first report of polysaccharides with immunoactivation in 1917 [9]. The antitumor activity of mushrooms was first demonstrated by Lucas *et al.* in1957 [10]. Only at the end of the 1960s eastern and western scientists started to investigate the mechanisms of the health effects of mushrooms. The first successful research discovered the antitumor effects of hot water extracts from several mushroom species [11]. The main active components were proved to be polysaccharides, specifically β-D-glucans. Chihara and his co-workers [12] isolated from the fruiting bodies of shiitake a water-soluble antitumor polysaccharide, which was named 'lentinan' after the generic name of this mushroom. Lentinan demonstrated powerful antitumor activity; preventing chemical and viral tumor development in mice and experimental models [13, 14]. Polysaccharides from mushrooms do not attack cancer cell directly, but produce their anti-tumor effects by stimulating macrophages such as natural Killer cells (NK-cell), T-cell, B-cell and macrophage-dependent immune system responses. Stimulating this aspect of the immune system it helps to protect against cold, flu and infections of any kinds. Several β-Glucans [15, 16] and α-Glucans [17] are widely used as antitumor and immunomodulating agents. These materials also act as biological response modifiers [16]. It is observed that structural features such as β- (1→3)-linkages in the main chain of the glucan and additional β-(1→6) branch points are needed for strong antitumor action. β-Glucans containing mainly (1→6)-linkages have less activity. High molecular weight Glucans appear to be more effective than those of low molecular weight [18-20]. Antitumor polysaccharides may have other chemical structures, such as hetero-β-glucans [21], heteroglycan [22], β-glucan-protein [23], α-mano-β-glucan [21], α-glucan-protein [21] and heteroglycan-protein complexes [24]. It is most important that polysaccharides having triple-helical structure show immune-stimulating activity.

Linear low molecular weight α (1→4) Glucans act as immunomodulatory and anticancer properties [25, 26]. Mushroom polysaccharides have immense utility, but most important aspect of mushroom polysaccharides is their immunomodulatory [27], anti-tumor and anti-cancer [28] effect as well. Several mushroom polysaccharides are widely used and commercialized worldwide as anti-cancer agents for therapeutic purpose. *Lentinus edodes* (Lentinan) [29] *Schizophyllan commune* (Schizophyllum) [29] *Agaricus blazei* (Agarican) [29, 30] *Ganoderma lucidum* (Lingzhi) [31, 32] *Grifola frondosa* (Maitake) [33] have been commercialized and used clinically as anti-tumor agents. Polysaccharides belong to a structurally diverse class of macromolecules, where polymers of monosaccharide residues are joined to each other by glycosidic linkages. It is noteworthy that, in comparison with other biopolymers such as proteins and nucleic acids, polysaccharides offer the highest capacity for carrying biological information because they have the greatest potential for structural variability. The nucleotides in nucleic acids and the amino acids in proteins can interconnect in only one way whereas the monosaccharide units in polysaccharides can interconnect at several points to form a wide variety of branched or linear structures [34]. The purpose of this paper is to review the isolation, purification, structural characterization and bioactivities of mushroom polysaccharides.

2. Isolation and purification of polysaccharides

To determine the exact structure of the polysaccharide it is of prime importance to isolate the pure polysaccharide as much as possible. Different techniques like chromatography, ultra centrifugation, dialysis, precipitation and re-precipitation are adopted for this purpose.

For structural analysis the polysaccharide should be homogeneous. Numerous methods have been employed for purification; they involve fractional precipitation, selective precipitation with detergents or metal ions, chromatographic techniques, electrophoresis and ionophoresis are used to determine the homogeneity of the polysaccharide isolated. The technique of column chromatography [35] is widely used for resolving a mixture of polysaccharides having different molecular weights. Sephadex and Sepharose gel are extensively employed in these cases.

3. Structural Analysis of polysaccharides

The structures of most bioorganic compounds, especially polysaccharides are more complicated than the structures of many of the organic compounds. Determinations of the structures of polysaccharides involve purification procedures, monosaccharide analysis, linkage analysis, anomeric configuration determination and identification of the sequence of sugar residues.

3.1. Sugars compositions and their mode of linkages

Paper chromatographic methods [36, 37] using different solvent system are used for separation and identification of different sugars in the hydrolysate of polysaccharides. The absolute configurations of monosaccharides, obtained from the hydrolyzed polysaccharide, are determined by the modified methods [38, 39]. Sugars in the hydrolysate of the polysaccharides are converted into their alditol derivatives and analyzed using gas-liquid-chromatography [40]. Linkage analysis is performed by methylation studies [41]. The method consists of complete methylation of polysaccharides followed by acid hydrolysis and identification of methyl sugar liberated. Identification and estimation of different methyl sugar obtained from the fully methylated polysaccharide can be done using GLC and mass spectroscopy (GLC-MS) [42]. For GLC-MS studies the sugar and their methylated derivatives are converted into their alditol acetates.

Periodate oxidation studies on polysaccharides and oligosaccharides provide supporting evidence for the structure of the repeating unit deduced from methylation studies. Vicinal diol groupings react with the oxidant, splitting carbon-carbon bond and forming a dialdehyde. The reducing sugar of a polysaccharide yields formaldehyde or formic acid depending upon the position of linkages of sugar unit whereas the non-reducing end groups yield formic acid. The limitation of periodate oxidation reaction is that it does not stop at the end point where the aldehydes groups are formed but proceeds causing non stoichiometric over oxidation. The rate of over oxidation is slower compared to that of glycol-splitting reaction and it can be controlled by buffering the reaction mixture and carrying out the reaction at low temperature.

3.2. Chemical degradation

Smith [43] degradation studies are also applied for identification of the position of linkages of sugar units in certain polysaccharides. Partial hydrolysis studies are commonly used for determining the sequence of sugar units present in a polysaccharide. In this method the oligosaccharide obtained, are separated using chromatographic techniques. Characterization of these oligosaccharides is one of the supporting evidences to identify the exact sequence of sugars residues present in the polysaccharide.

3.3. Nuclear Magnetic Resonance study

The sequences of sugar residues present in polysaccharides and oligosaccharides are confirmed by the 1D and 2D NMR (^{1}H, ^{13}C, 2D-COSY, DQF-COSY, TOCSY, NOESY, HSQC, HMBC, etc.) analysis. Integration of the anomeric proton resonances offers an initial estimation of the number of different monosaccharide residues present in the polysaccharide. The anomeric proton resonances are found in the range 4.4-5.5 ppm. The remaining ring protons resonances are found in the range 3–4.2 ppm. The anomeric configurations of sugars are initially determined using coupling constant value ($J_{H\text{-}1,H\text{-}2}$) from ^{1}H NMR spectrum. The vicinal coupling constant between the anomeric H-1 and the H-2 indicates the relative orientation of two protons. If they are both in an axial configuration in pyranose structures, a large coupling constant (7-8 Hz) is observed, whereas if they are equatorial-axial, this is smaller ($J_{H\text{-}1,H\text{-}2}$ ~ 4 Hz), and for axial- equatorial or equatorial- equatorial oriented protons even smaller coupling constants are observed (<2 Hz) [44]. Different sugars are identified using their characteristic *J* coupling pattern. β-D-Glc*p* has characteristics coupling constants: $J_{H\text{-}1,\ H\text{-}2}$ ~ 7.9 Hz; $J_{H\text{-}2,\ H\text{-}3}$ ~ 9.5 Hz; $J_{H\text{-}3,\ H\text{-}4}$ ~9.5 Hz and $J_{H\text{-}4,\ H\text{-}5}$ ~ 9.5 Hz. For α-D-Glc*p*, $J_{H\text{-}1,H\text{-}2}$ ~ 3.6 Hz; $J_{H\text{-}2,H\text{-}3}$ ~ 9.5 Hz; $J_{H\text{-}3,\ H\text{-}4}$ ~9.5 Hz and $J_{H\text{-}4,H\text{-}5}$ ~ 9.5 Hz are generally observed. In case of α-D-Gal*p*, $J_{H\text{-}1,\ H\text{-}2}$ ~ 3.9 Hz; $J_{H\text{-}2,\ H\text{-}3}$ ~ 10 Hz; $J_{H\text{-}3,\ H\text{-}4}$ ~3.8 Hz and $J_{H\text{-}4,\ H\text{-}5}$ ~ 1 Hz, and for β-D-Gal*p*, $J_{H\text{-}1,\ H\text{-}2}$ ~ 8 Hz; $J_{H\text{-}2,\ H\text{-}3}$ ~ 10 Hz; $J_{H\text{-}3,\ H\text{-}4}$ ~3.8 Hz and $J_{H\text{-}4,\ H\text{-}5}$ ~ 1 Hz are observed. The α-D-Man*p* sugar is identified by observing $J_{H\text{-}1,\ H\text{-}2}$ ~ 1.8 Hz; $J_{H\text{-}2,\ H\text{-}3}$ ~ 3.8 Hz; $J_{H\text{-}3,\ H\text{-}4}$ ~10 Hz and $J_{H\text{-}4,\ H\text{-}5}$ ~ 9.8 Hz and β-D-Man*p* is also showed almost same type of coupling pattern. The α-L-Fuc*p* sugar shows similar coupling pattern of α-D-Gal*p* along with $J_{H\text{-}5,\ H\text{-}6}$ ~ 6.3

Hz, and characteristic chemical shifts at δ_{H-6} ~ 1.1 ppm (CH_3) and δ_{C-6} ~ 16.3 ppm (CH_3) are observed. In this connection most important one band ^{13}C - ^{1}H coupling constants can be used to determine the anomeric configuration perfectly. $^{1}J_{C-1,H-1}$ ~170 Hz indicates an α- anomeric sugars configuration, whereas $^{1}J_{C-1,H-1}$ ~160 Hz indicates a β- anomeric sugar configuration [45]. Since the difference in $^{1}J_{C-1,H-1}$ value between two α- and β- anomeric configurations is generally 10 Hz, so it is most effective for determination of anomeric configurations of sugars [46-48].

Table1: Representative NMR chemical shifts for various groups of polysaccharides

^{1}H	ppm	^{13}C	ppm
C-CH_3	1.1-1.3	C-CH_3	16-18
(CH_3)COO	1.8-2.2	(CH_3)COO	18-22
O-CH_3	3.3-3.5	O-CH_3	55-61
H_2-H_6	3.2-4.5	CH_2OH, CH_2OR, C_2-C_5	57.7-64.7, 66-70, 65-87
H1 (ax)	4.3-4.8	C1 (ax-O, red) C1 (Ketose) C1 (ax-O, glyc)	90-95, 98-100, 98-103
H1 (eq)	5.1-5.8	C1 (eq-O,red) C1 (eq-O, glyc) C1 (fur) COO	95-98, 103-106, 103-112, 170-180

All proton signals (H-1 to H-6) of sugar residue are identified using 2D-DQF-COSY and TOCSY experiment.

The sequences of glycosyl residues are determined from NOESY (Nuclear Overhauser Enhancement Spectroscopy) and ROESY (Rotating frame Overhauser Enhancement Spectroscopy) experiment. NOE connectivities are often observed between the anomeric protons of a particular sugar residue to proton of the other sugar residues that is glycosydic linked to the former sugar residue. The presence of an inter residue NOE defines the glycosydic linkage and provides sequence information of a polysaccharide. In NOESY spectrum numerous peaks are obtained. Therefore, the first step in analyzing NOESY spectrum is to eliminate those uninteresting peaks comparing them to DQF-COSY and TOCSY spectrum.

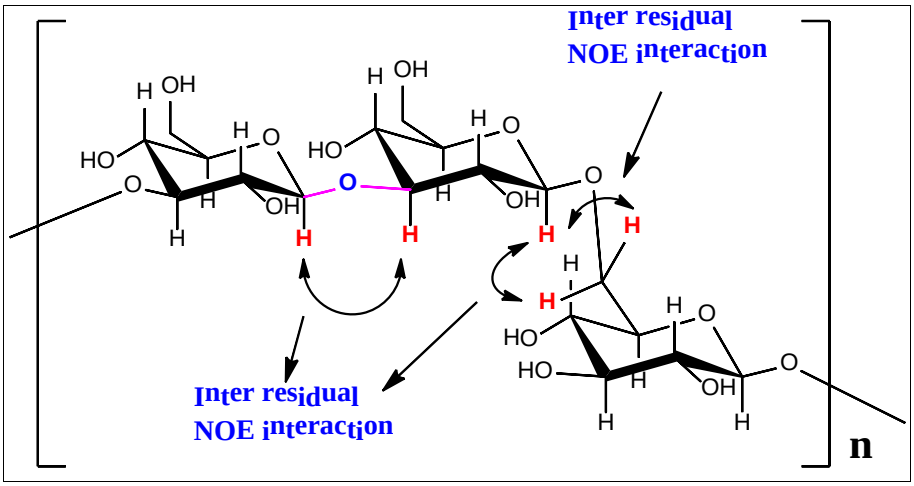

Fig 1: Intersexual NOE contacts observed in NOESY spectrum for (1→3), (1→6)-β-glucan.

The ^{13}C NMR spectroscopy is most important for structural determination of polysaccharide. All the ^{13}C chemical shifts of the sugar residues are here assigned from the individual proton signals assigned from DQF-COSY and TOCSY spectrum as they are directly correlated. The HSQC experiment is very helpful to predict the all carbon signals of each sugar residues present in the polysaccharide. HMBC experiments establish multiple-bond correlation through the glycosidic bonds, and this together with NOESY experiments provides necessary information on linkages and sequences of a polysaccharide.

4. Bioactive properties

Mushrooms useful [29] against cancers of the stomach, esophagus, lungs, etc. are known in China, Russia, Japan, Canada and United States. Three polysaccharide based carcinostatic [49] (immunotherapeutic) agents, Krestin, Lentinan and Sonifilan, have already been developed from mushroom. These are used currently in the treatment of cancer of the digestive organs, lung and breast, as well as cancer of the stomach and cervical cancer respectively. Mushroom polysaccharides are also used as multipurpose medicines that are not only carcinostatic but also anti-inflammatory, antiviral (against AIDS), hypoglycemic and antithrombotic [49].

Lentinan, isolated from *Lentinus edodes* is a β-(1→3), β-(1→6) glucan. There is an immense literature related to the anticancer effect of lentinan on animal and human carcinomas. It was first isolated and studied by Chihara *et al.* who demonstrated that its antitumor effects were greater than other mushroom polysaccharides [50]. There have been numerous clinical trials of lentinan in Japan, and the drug is now manufactured and sold by several pharmaceutical companies. Lentinan has been successfully used in prolonging the overall survival of cancer patients, especially those with gastric and colorectal carcinomas [51-53]. Schizophyllan is the polysaccharide derived from the mushroom *Schizophyllum commune*. It has been shown to be effective in sarcoma-180 tumor xenographs [29]. Various clinical trials have been carried out in Japan. Early clinical studies with schizophyllan in combination with conventional chemotherapy (tegafur or mitomycin C and 5-fluorouracil) in a randomized controlled study of 367 patients with gastric cancer showed significant increase in median survival [54]. Recently schizophyllan has also been shown to increase overall survival of patients with head and neck cancers [55]. In a randomized controlled study

of schizophyllan in combination with radiotherapy, showed that it significantly prolonged the overall survival of stage II cervical cancer patients [56, 57]. Schizophyllan is currently produced commercially by several Japanese pharmaceutical companies. Several studies have shown that β-D-glucan derived from another mushroom *Grifola frondosa* have strong antitumor activity in xenographs [58]. More recently, a highly purified extract, β-(1→3),(1→6)glucan (Grifron-D, GD) has become available. GD has considerable immunomodulating and antitumor activities in animal models, and is orally bioavailable [59]. Maitake D-fraction and crude Maitake powders have demonstrated remarkable inhibition of metastasis in a mouse model, especially in the prevention of hepatic metastases [60]. GD has been shown to have a cytotoxic effect on human prostate cancer cells (PC9) *in vitro*, possibly acting through oxidative stress, and causing 95% cell death by an apoptosis [61]. Polysaccharide isolated from *Agaricus blazei* was shown to be an immune system stimulant, promoting body's natural defense mechanisms to fight a variety of infectious agents and conditions, including cancer. The immunostimulating activity and antitumor action of *Agaricus blazei* extracts were investigated in different laboratory models, including Sarcoma 180 and fibrosarcoma tumor-bearing mice [62-67]. Seven polysaccharide fractions obtained from *Agaricus blazei* fruit bodies were demonstrated to have antitumor activity. Analyses of physico-chemical properties of water-soluble polysaccharide fractions having high antitumor activity showed that their main components were β-(1→6), β-(1→3)-glucan, acidic β-(1→6), α-(1→4)-glucan, and acidic β-(1→6), α-(1→3)-glucan [68]. A new antitumor polysaccharide, β-(1→2); β-(1→3)-glucomannan [69] active against Sarcoma 180 was recently separated from liquid cultured mycelium of *Agaricus blazei*. An antitumor glucan was isolated from the neutral polysaccharide fraction of a hot-water extraction of the edible mushroom *Pleurotus ostreatus* [70].

Mushroom polysaccharides also act as dietary fibers [71]. Dietary fibers are resistant to digestion and absorption in human small intestine with partial or complete fermentation in the large intestine [72]. They promote beneficial physical effects such as laxation, blood cholesterol attenuation and blood sugar attenuation. Mushrooms contain dietary fibres belonging to β-glucans, chitin, and heteropolysaccharides as much as 10% -50% in the dried matter. Most of the active polysaccharides water soluble, or insoluble, isolated from mushrooms can be classified as dietary fibers. The mushroom polysaccharides absorb bile acids or other hazardous materials in the intestine, and thus decrease the chance of carcinogenic and other poisoning. Mushroom polysaccharides may work effectively to prevent cancer of the colon and rectum [73].

5. Structural features of mushroom polysaccharides exhibiting bioactivities

Mushroom polysaccharides are present mostly as glucans with different types of glycosidic linkages, such as (1→3), (1→6)-β-glucan, (1→3)-α-glucans, and some are true heteroglycans. The others mostly bind to protein residues as PSP complexes [74].

The main source of antitumor polysaccharides appears to be fungal cell wall polysaccharides. Besides the well-known antitumor β-(1→3)-glucans, a wide range of biologically active glucans with other structures have been described. These polysaccharides have linear or branched molecules in a backbone composed of α-or β-linked glucose units, and they contain side chains that are attached in different ways. Polysaccharides with antitumor action differ greatly in their chemical composition, configuration, and physical properties. Antitumor activity is exhibited by a wide range of glycans extending from homo-polysaccharides to highly complex hetero-polysaccharides [75]. Differences in activity can be correlated with solubility in water, size of the molecules, branching rate and form. Although it is difficult to correlate the structure and antitumor activity of complex polysaccharides, some relationships can be drawn. It is obvious that structural features such as β-(1→3) linkages in the main chain of the glucan and additional β-(1→6) branch points are needed for antitumor action. β-glucans containing mainly (1→6)-linkages have less activity. High molecular weight glucans appear to be more effective than those of low molecular weight [76-78]. However, obvious variations in antitumor polysaccharides have also been noted. Antitumor polysaccharides may have other chemical structures, such as hetero-β-glucans [79] heteroglycans [80], β-glucan-protein [81], α-manno-β-glucan [79], α-glucan-protein and heteroglycan-protein complexes [82]. Linear low molecular weight α-(1→4)-glucans can also exhibit immunomodulatory and anticancer properties [83, 84]. Triple-helical conformation of β-(1→3)-glucan is known to be important for their immune-stimulating activity. When lentinan was denatured with dimethyl sulfoxide, urea, or sodium hydroxide, helical structure was lost while primary structure was not affected, but tumor inhibition properties were lowered with progressive denaturation [85]. The investigation of schizophyllan [86, 87] also gave same result which confirm the correlation between antitumor activity and triple helix structure.

6. Conclusion

Polysaccharides belong to a structurally diverse class of macromolecules, in which polymers of monosaccharide residues are joined to each other by glycosidic linkages. The different activities of the polysaccharide depend on the size of the molecules, solubility in water, branching rate, and conformation. For this, it is needs to determine the probable structure of the polysaccharides isolated from mushrooms. The future challenge is to deduce the 3D structure of polysaccharides and the structure-function-relationship.

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