VARIATION OF BIOACTIVE LENTINAN-CONTAINING PREPARATIONS IN *LENTINULA EDODES* STRAINS AND STORED PRODUCTS

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ABSTRACT

Lentinan, a ß-(1→3)-D-glucan isolated from the common edible mushroom, *Lentinula edodes* (Shiitake), is a biologically active macromolecule with a potential medical application towards immune functions. The immunomodulatory activities of ß-glucans depends on their degree of branching, conformation and the inter- and intra-molecular association of the polysaccharide. Up to now, there is a lack of understanding the relationship between the structure and function of lentinan and no quality controlling methods are available to analyse bioactivity of lentinan containing supplements or for the Shiitake mushrooms. Therefore, we attempted to clarify the relation between structural details and biological responses of lentinan extracts isolated from fresh and stored mushrooms.

From freshly harvested fruiting bodies, the yield of crude lentinan extract varied from 260 to 825 mg/100g fresh weight. After 4 days of cold storage, the variation in crude lentinan extract content was more pronounced among the six strains analysed. As a general tendency, the yield decreased during storage with the exception of one strain for which lentinan content appeared to increase during storage.

The bioactivity of the crude lentinan extracts was studied using the RAW264.7 macrophage cell line and the ability to modulate the NO production of LPS challenged. Results indicated that the crude lentinan extracts could inhibition of LPS-induced NO production but again some strains showed higher immune modulatory effects than others. Size exclusion chromatography (SEC) was applied to examine molecular weight distribution of the crude lentinan extracts. It appeared that in crude lentinan extracts of some of the strains, the polysaccharide consisted of a different conformation or modification of lentinan or contained other polysaccharides. In general, the presence and ratio of some of the different polysaccharide forms that could be distinguished by SEC analysis are correlated to *in vitro* bioactivity.

In conclusion, our study resulted in a broader insight into structure-function relationship and the possible influences of strain, growth and storage condition on bioactivity of Shiitake lentinan. The SEC and bioactivity analyses together presented a possible direction of quality control for lentinan-containing products. The standardisation of lentinan-containing bioactive products should include the background knowledge of strain, and of growth and storage conditions of the mushroom.

INTRODUCTION

Lentinula edodes, the Shiitake mushroom, is well-known for its health-promoting effects, such as antitumor, hypocholesterolemic actions, antimicrobial and antioxidants potentials [1,]2]. Lentinan isolated from Shiitake is recognized as being an effective biological response modifier [3]. The molecular formula of lentinan is $(C_6H_{10}O_5)_n$ with a mean molecular mass of 500 kDa [5]. The backbone structure of lentinan has been reported as a ß-1,3-D-glucan backbone, branched with ß-(1-6)-glucans [4] which show a right-handed triple helix [5].

It is known that the molecular weight, degree of branching, conformation, and intra- and intermolecular association of the polysaccharide chains are important for their biological responses [6].

Previous studies have shown that the biological response of lentinan against infection is host mediated and due to activation of the innate and adaptive immune responses. For the activation of appropriate immune responses, pattern recognition by receptors is important. An important receptor for ß-glucans, including lentinan, is dectin-1 [7]. Dectin-1 is mainly expressed on the surface of macrophages, neutrophils, dendritic cells (DC) and on some T-cells [8].

Several studies reported effects of lentinan on cytokine production of macrophages or monocytes in both mice and human, for instance, production of TNF-α, IL-1β and cytotoxic- or nitric oxide (NO) activation [9]. NO is a noxious, stable and free radical gas which plays an important role in the functions of macrophages. It has been reported that mouse macrophages stimulated with antitumor polysaccharides produced NO [11] and also macrophages stimulated with lentinan released NO [12]. Thus it seems that enhancement of NO production through macrophages may reflect the antitumor activity of lentinan [10]. In addition, Miniato [13] concludes from his investigations that the quality of the Shiitake as a functional food is correlated to the lentinan content. Especially during storage of Shiitake, a decrease in lentinan content was observed, which was caused by enzymatic degradation through exo-glucanase. There are no specified reports yet on the impact of strain or storage conditions on the composition, content and immunomodulating effect of lentinan. In this study, we analyzed the effect of storage on composition changes and immunomodulating effects of lentinan in different Shiitake strains.

MATERIAL AND METHODS

Materials. Purified lentinan (4 mg/ml) was kindly provided by Ajinomoto Co.

Mushroom sample preparation. The shiitake mushrooms strains were selected from the collection of Plant Research International (PRI), Wageningen University and Research Centre. The different Shiitake strains were grown on artificial substrate under Dutch commercial breeding circumstances.

At PRI, Sawdust substrate bags (Hesse 2,8 kg) were inoculated with 30 ml spawn. These spawn was prepared by inoculating pure cultures on sterilized sorghum grain. The bags were incubated using the following climate parameters: 20°C, 1500ppm CO_2 and 90% RH. After 6-11 weeks of vegetative growth, depending on the strain, the plastic was removed from the blocks and the blocks were placed in a production room. The conditions for fruiting body production were 16°C, 1000ppm CO_2, 90% RH and 12 hours light a day. Fruiting body production started, again strain dependent, after 6 to19 weeks of vegetative growth. Immediately after harvesting, the fruiting body's were packed in moisturized boxes and stored for 4 days at 7°C. After 4 days storage, as well as directly after harvest, mushrooms were sectioned into cap and stipe part, frozen with liquid nitrogen and stored at -80°C. Only the cap tissue is discussed in this article because of limitations in available stipe tissue.

Extraction of crude lentinan. The extraction of crude lentinan was modified from the method developed by Yap and NG [14]. To 100 g of frozen cap tissue, boiling water was added with a solid:liquid ratio of 1:3. The samples were homogenized with an Ultrathurax for 1 min and boiled for 3h under continues stirring on a heating plate. After cooling down the extracts to room temperature, the lentinan was precipitated by adding one volume of 95% ethanol followed by an incubation step of 16h at 4°C. The samples were centrifuged at 5000 rpm (Beckman) for 20 min at 4°C. The pellet was snap frozen in liquid nitrogen and lyophilized in a freeze-dryer. One volume of hot water (60°C) was slowly added to the lyophilized pellet and the solute was homogenized using an Ultrathurax for 1 minute at full speed. Subsequently, the homogenate was boiled for 8h under continues stirring, stored overnight at 4°C and centrifuged at 5000 rpm for 20 min at 4°C. The supernatant was collected and precipitated overnight with 1 volume of 95% ethanol. The precipitate was collected by two centrifugation steps at respectively 5000 and 7000 rpm for 20 min at 4°C and subsequently lyophilized. The obtained crude lentinan powder was further dried in an oven at 60°C for 1 day. The samples were weighed (concentration lentinan / g tissue) and stored in a desiccator prior to further analysis.

Molecular characteristics of crude lentinan. To investigate the purity of crude lentinan extract, the protein content using a Bradford method [16] and the total phenolic compounds, with some modifications [17], were determined. Briefly, 5 mg of each extract was dissolved in 1 ml methanol while stirring on a boiling plate at 60°C for 24 hours. To 100µl of crude lentinan extract, 500µl Folin-Dennis reagent (Sigma) and 1ml of saturated sodium carbonate solution were added. A standard curve was prepared using tannic acid (0.1-1.0 mg/100µl), with the addition of 500µl of Folin-Densis reagent (Sigma) and 1 ml of saturated sodium carbonate solution. All the samples were centrifuged and the absorbance values from the clarified supernatants were measured using spectrophotometer at 760nm. The total phenolic content was calculated based on equivalent to tannic acid (ETA). Size-exclusion chromatography (SEC) was used to determine the molecular weight and viscosity of the samples. The SEC measurements were carried out on a triple detection GPC/SEC (Viscotek). Crude lentinan samples (3 mg/ml) were dissolved in dimethylacetamide with 0.5% lithium chloride (DMAc/0.5%LiCl) which was also used as eluent. A homogeneous solution was obtained through continuous stirring at 60°C for 48 hours. All samples were filtered by a 0.45 µM glass microfiber filter (Whatman) before injection (100µl) onto the SEC GMHHR-M + Guard column. The flow rate was 1.0 ml/min. Eight narrow molecular weight pullulan standards in the range 5.8 – 1660 kDa (Shodex standard P82, Showa Denko) were used to calibrate the columns. The TriSec software program version 4 was used for the acquisition and analysis of Viscotek data.

Effect of crude lentinan on NO-production. The effect of crude lentinan on NO production was evaluated. RAW 264.7 cells, a murine macrophage cell line, was cultured in RPMI 1640 medium (Gibco) supplemented with 100 U/ml penicillin, 100µg/ml streptomycin and 10% heat-inactivated fetal bovine serum (Invitrogen). Cells were grown at 37°C under an atmosphere of 5% CO_2. RAW 264.7 cells (10^6 – 10^7 cells/well in 96-well culture plates) were incubated at 37°C (5% CO_2), with or without 10µg/ml LPS (sigma), and complemented with 100 µg/ml crude lentinan extracts for 48 hours. After the incubation period, NO production was determined using a colorimetric test based on the Griess reaction [15]. Briefly, 50 µl of cell supernatant was mixed with 50 µl Griess reagent (Sigma) and the mixture was incubated at room temperature for 10 min. The nitrite concentration was determined by measuring the absorbance at 540 nm in an automated plate reader (Multiskan Spectrum Thermo Labsystems) using the standard curve of $NaNO_2$. The results were expressed as relative percentage of NO production compared to LPS control (100%) with subtraction of the media control.

RESULTS AND DISCUSSION

Variation in lentinan content in different Shiitake strains during storage. Mostly all consumable mushrooms are distinguished as healthy, functional foods, and well-known to contain some types of immunomodulating polysaccharides, specific the polysaccharide called lentinan. In order to investigate the influence of genetic background and the effects of storage on the lentinan content and bioactivity, 13 different Shiitake strains were cultivated and harvested under the same growth conditions. The production was performed only once. As some strains had a very low fruiting body production not all of the analyses could be performed in full matrix (both fresh and stored at 7°C) or in duplicates. Table 1 shows the changes in crude lentinan content from six Shiitake strains with highest fruiting body production, directly after harvest (fresh) and after 4 days of cold storage at 7°C. The content of lentinan of fresh harvested Shiitake varied between 260 and 824 mg/100g fresh weight (fw). Mizuno [22] observed that the content of lentinan did not change drastically during storage at 5°C for 7 days. In this study, we noticed that the decline of crude lentinan content was different per strain. The crude lentinan content of strain Mes02094 decreases minimal (260 to 226 mg/100gr fw). While in strain Mes02007 the crude lentinan content decreased drastically during storage (824 to 229 mg/100g fw). Thus, we can concluded that change in lentinan content does not only depend on storage conditions but also on the Shiitake strain.

Table 1: Lentinan content from different Shiitake strains after harvest and after 4 days at 7°C

Strain	lentinan content mg/100gr fw	
Mes number	fresh	Storage
02007	824	229
02010	727	1478
02054	260	226
02094	244 ± 42	166 ± 4
02121	332 ± 16	229 ± 18
11775	448	385

Molecular characteristics of the crude lentinan containing extracts. Prior to the evaluation of bioactivity of the crude lentinan extracts, the purity of the extracts was investigated. It is known that phenolic compounds (at the range of 16-500µM) cause an inhibitory activity on NO production in LPS activated macrophages of more than 50% NO production [21]. Therefore, the content of phenolic compounds in the crude lentinan extracts was analyzed as show in Table 2. All extracts, contained before and after storage, very low levels of phenolic compounds (< 2µM). As a result, we presumed that the present phenolic compounds in the crude lentinan extracts had no effect on the stimulation/inhibition of NO production. Likewise, the protein levels (Table 2) in the crude lentinan extracts were very low and therefore the present proteins should give no disturbing effect on the bioactivity assay.

Size exclusion chromatography (SEC) was applied to study the composition of the crude lentinan extracts. SEC results revealed that the crude lentinan extracts consisted of several peaks, based on Molecular weight (Mw) (Table 2) and the retention time (data not shown). These peaks correspond to the protein attached triple helix chains and to the fragments of triple helix with a high molecular weight and single chains having low molecular weight [23].

Table 2: Molecular characteristics of crude lentinan extracts and pure lentinan.

Strain Mes number	Phenolic compounds %	Protein %	Mw peak 1 $x10^5$	Mw peak 2 $x10^4$	Mw peak 3	Mw/Mn peak 1	Area peak 1 mV/ml	Area peak 2 mV/ml	Area peak 3 mV/ml
02007-fresh	0.10 ± 0.02	0.75 ± 0.08	2.21	1.27	412	1.9	93.41	15.41	1.75
02010-fresh	0.09 ± 0.04	0.66 ± 0.03	2.87	1.24	431	2.1	85.92	7.71	1.17
02054-fresh	0.12 ± 0.02	0.75 ± 0.02	2.73	1.34	441	2.0	26.54	16.36	3.55
02094-fresh	0.14 ± 0.01	0.54 ± 0.02	2.00	1.26	445	1.6	25.62	10.29	2.08
02121-fresh	0.19 ± 0.02	0.86 ± 0.02	2.12	1.26	462	1.7	62.68	15.36	2.26
11775-fresh	0.17 ± 0.07	1.21 ± 0.04	2.03	1.35	515	1.6	78.30	14.93	2.56
02007-storage	0.26 ± 0.01	1.23 ± 0.00	3.44	1.50	396	2.0	7.13	33.15	5.84
02010-storage	0.06 ± 0.01	0.77 ± 0.00	2.47	1.35	502	1.7	117.7	7.71	0.78
02054-storage	0.32 ± 0.02	0.99 ± 0.03	2.21	1.34	439	2.0	22.35	21.75	4.56
02094-storage	0.19 ± 0.01	0.61 ± 0.01	2.97	1.20	429	2.6	30.20	24.25	5.25
02121-storage	0.18 ± 0.00	1.05 ± 0.04	2.45	1.78	452	1.8	24.79	23.64	4.22
11775-storage	0.24 ± 0.02	1.64 ± 0.03	2.93	1.41	525	2.4	51.18	21.72	3.98
lentinan	0.01 ± 0.01	nd	4.00	1.00		2.6	39.33	7.94	

It was estimated that the lentinan extract derivatives might be modified during the storage of the fruiting bodies, extraction of the lentinan and storage of the extracts, thus causing the degradation of polysaccharides. Cold storage could induce further changes, probably through depolymerisation and oxidation of the polysaccharide. SEC analyses identified that pure lentinan exhibited two peaks with a Mw of 4.0 x 10^5 and 1.0 x 10^4 (Table 2). The Mw of pure lentinan has been determined to be $9.5x10^5$-$10.5x10^5$ [4] and later found to be $2.03x10^5$-$8x10^5$ by SEC analyses [19, 20]. From our results, the Mw of peak1 found in all crude lentinan extracts either from fresh or cold stored fruiting bodies, corresponded with the expected Mw in literature. The Mw from the pure lentinan (received from Ajinomoto and used as reference) is approximately 1.5 times higher than the Mw of the isolated lentinan extracts, probably caused by the conformation of the lentinan due to the isolation and purification procedure used.

The polydispersity (Mw/Mn) of the investigated crude lentinan extracts were found to be between 1.6 and 2.6 (Table 2) and are similar to the finding of Zhang's group [18, 24]; they indicated that the polydispersity of lentinan fractions were between 1.8 and 2.7. In general, the polydispersity of the stored shiitake was higher than from fresh Shiitake. From this we can conclude that partially, the backbones of these modified polysaccharides were more degraded than the polysaccharides in the fresh harvested Shiitake.

Modification of LPS induced NO production. The function of macrophages may be comprised by lentinan via two mechanisms: I) by cell-to-cell contact between macrophages and tumor cells and II) by the release of antitumor factors and mediators such as cytokines and NO [9]. When LPS was administered to RAW264.7 macrophages, the production of NO increased dramatically. To determine the suppressing abilities of crude lentinan on NO production, RAW macrophages were incubated with or without 10µg/ml LPS in combination with or without crude lentinan extracts (100 µg/ml). The inhibitory effect (IE) was expressed as the percentage of decrease in NO production as where $[NO]^a$ represents the NO concentration of cells supplemented with

lentinan and LPS minus $[NO]^b$, the NO concentration of cells supplemented with lentinan alone. $[NO]^c$ represents the NO concentration from LPS activated control macrophages.
$IE(\%) = 100 - ([NO]^a - [NO]^b) / [NO]^c * 100)$.

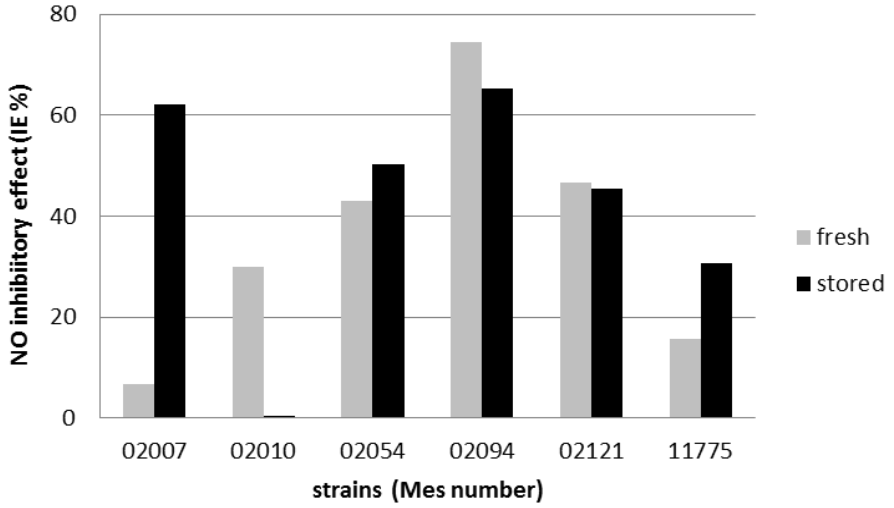

Figure 1: Inhibitory effect on NO production (%) of macrophages by crude lentinan extracts from fruiting body tissue of different strains Shiitake directly after harvest (fresh) and after storage of the fruiting bodies 4 days at 7°C (stored).

Cell viability was assayed to exclude the possibility that the inhibitory effects obtained from crude lentinan extracts might be caused by their cytotoxicity (data not shown). The inhibitory effects of the crude lentinan extract originating from fresh and cold stored fruiting bodies on NO inhibition in LPS activated macrophages is shown in Fig. 1. The crude lentinan extracts of all six strains demonstrate an inhibitory effect of NO production in macrophages, although the effects varied among strains. Crude lentinan extracted from fresh strain Mes02094 showed the highest NO inhibition (75%) while the extract from cold stored Mes2007 showed the least response (7%). Fresh and cold stored Mes02121 showed an equal NO inhibition response while for strains Mes02054, Mes02094 and Mes02121 a difference in response between fresh and stored mushroom extract was seen. The inhibitory effect of strain Mes02010 after cold storage was close to zero, while the inhibitory effect from Mes02007 drastically increased after the cold storage.

In conclusion, the consumption of the diet rich-lentinan –containing Shiitake- may reduce the production of nitric oxide caused by the oxidative stress, thus might increase the protective effects against cardiovascular and chronic inflammatory diseases. When testing the health effects of Shiitake mushrooms in intervention studies, strain and product freshness should be taken into account. Preferably, products should be standardized by using batch wise biochemical and bioactivity analysis.

Relationship between concentration, molecular characteristics and bioactivity of the crude lentinan extracts. The immunomodulating properties of lentinan, observed in NO producing macrophages, are related to the chemical composition, configuration and chain conformation, as well as their physical properties [6]. From our results we have tried to identify a relationship between characteristics of the isolated crude lentinan and NO inhibitory bioactivity. For instant, an exponential negative correlation between NO inhibitory effect and total lentinan content was observed (Fig. 2). This might indicate that higher concentrations of the polysaccharides could reduce the immune modulating competency of the polysaccharide. On the other hand, it could also indicate that, under some conditions, the content of lentinan in crude extracts were

overestimated resulting in the use of lower concentrations in the bioassay than expected based on weight basis.

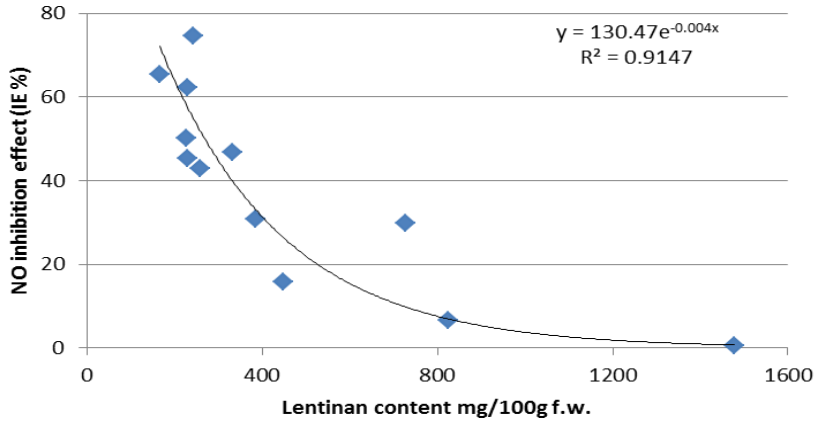

Figure 2: Relationship between the NO inhibition effect (%) and the crude lentinan content (mg/100g fw).

We conclude from that observation that the quantity of the crude lentinan in Shiitake extracts could not be used as a single factor for the immunomodulating capacity of Shiitake mushrooms.

Table 3: Correlation between NO inhibitor activity and peak area after SEC analysis.

R^2	Area peak 1	Area peak 2	Area peak 3	% NO inhibition
Area peak 1	1	-0.783**	-0.896**	-0.833**
Area peak 2	-0.783**	1	0.951**	0.506
Area peak 3	-0.896**	0.951**	1	0.629*
Area peak 2+3	-0.905**	0.935**	0.999**	nd
% NO inhibition	-0.833**	0.506	0.629*	1

p<0.01** , p<0.05*, n=12, nd = not detected

Apart from the lentinan-NO inhibition-relationship, the correlation between NO inhibitor activity and the peaks found after SEC analysis were studied (Table 3). A high interaction between peak areas (equivalent to concentration) was observed, e.g. between peak 1 and 3 in which the concentration of peak 3 increases while peak 1 decreases (R^2 =-0.896; p<0.01). Very likely, this is caused by conformational changes due to degradation. Peak 1, which also has been identified in the pure lentinan from Aijinomoto, as well as peak 3 correlates to the NO inhibition. High concentration of peak 1 in crude extracts relates to lower NO inhibition while increase in peak 3 concentration results in higher NO inhibition effects. Peak 2 showed less correlation with the NO inhibitory effect. However, the sum of peak 2 and peak 3 showed the highest positive correlation with NO inhibition (R^2= 0.905; p<0.01). Some of the strains (Mes 02007, 02054 and 02094) exhibited a fourth peak but the area of this peak was very small and revealed no correlation with the NO inhibitor activity.

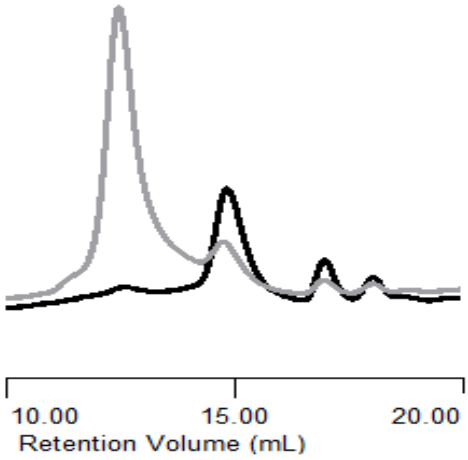

Figure 3: SEC spectra of strain Mes02007 directly after harvest (grey) and after storage of 4 days at 7°C (black).

In general, most strains did not show much change in peak composition when stored as fresh fruiting bodies. However, strain Mes02007 showed a remarkable change in peak area shift from peak 1 towards peak 2 and 3 accompanied by a high NO inhibition bioactivity (Fig. 3). This strain might therefore be an interesting genetic source for fresh Shiitake functional foods as this requires a high storability with remained bioactivity.

CONCLUSION

Future research should focus on science based evidence of lentinan to support our immune system, especially on a food product bases like using whole Shiitake mushrooms or food supplemented with isolated lentinan. These studies however should include standardized methods to verify the product bioactivity knowing the biological variation that can be caused by strain, storage, purification, process and probably still other factors which should be unraveled in order to develop reliable functional food products.

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