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Effects of Management Factors on the Concentration of a High Molecular Weight Polysaccharide Fraction from Log-Grown Shiitake Mushrooms (*Lentinula edodes* (Berk.) Pegler)

Tom E. Kimmons,[†] Mark Phillips,[‡] and David Brauer*[,§]

[†]Shirley Community Development Corporation, 366 Brown Road, Shirley, Arkansas 72153, [‡]Shiitake Mushroom Center, 366 Brown Road, Shirley, Arkansas 72153, and [§]Conservation and Production Research Laboratory, ARS-USDA, P.O. Drawer 10, 2300 Experiment Station Road, Bushland, Texas 79012

Shiitake mushrooms have a reputation as a healthy food. Growers may be able to use the presence of health-promoting constituents as a marketing tool to promote sales of their products for premium prices. There are few reports on the effects of management protocols for log-grown shiitakes on the concentrations of constituents to guide growers. This paper summarizes several studies that examined the effects of shiitake strains, mushroom cap development, and length of saprophytic association on the concentrations of a high molecular weight polysaccharide fraction that includes lentinan (HMWP). Concentrations of HMWP in mushrooms varied as much as 8-fold during fruiting among the 12 strains tested in these studies. Results also indicate that the concentrations of HMWP in shiitake mushrooms are influenced by the fungal phenotype and the characteristics of the environment. General trends showed that (1) mushrooms harvested at more immature stages of development (during bud break or before veil break) tended to have higher concentrations of HMWP and (2) the initial harvests of mushrooms from an inoculated log tend to have higher concentrations of HMWP than subsequent harvests. Results suggest that growers interested in maximizing the HMWP content of their mushrooms should use shiitake strains NN-430 and 569-430.

KEYWORDS: Lentinan; fungal strain; environment; mushroom maturation; saprophytic association; polysaccharide

INTRODUCTION

Shiitake (*Lentinula edodes* (Berk.) Pegler) mushroom production in the United States has expanded greatly since its start about 30 years ago. Current annual shiitake production exceeds 9,000,000 pounds (*1*). Worldwide sales of medicinal mushroom products were estimated to exceed $ 10 billion (U.S.) in 2000 (*2*). Worldwide sales of medicinal mushroom products have probably increased since 2003, when skin care products made from shiitakes were introduced into the marketplace (*3*).

Fungal homogeneous and heterogeneous polysaccharides have been identified as promoting human health (*4*). One of the suspected effects of fungal glucans is immune system stimulation (*5*−*7*). Sales of mushroom-based immune boosters increased approximately 3-fold between 2002 and 2003 (*8*). Lentinan has been identified as a health-promoting, water-soluble β-glucan from shiitakes (*5*, *7*). There is evidence that intravenous injections and ingestion of lentinan promote human health (*9*) and stimulate the immune system (*5*−*7*). However, the health effects of lentinan ingestion have not been studied as extensively as responses to lentinan intravenous injections. Shiitakes may contain other glucans with health-promoting properties, but specific polysaccharides have not been identified yet.

*Corresponding author [telephone (806) 356-5769; fax (806) 356-5769; e-mail david.brauer@ars.usda.gov].

Lentinan consists of a β-(1−3)-linked glucan backbone with two β-(1−6)-linked glucose side chains for every five β-(1−3)-linked glucose residues and has a molecular weight of about 400 kDa (*10*). Lentinan is readily soluble in water but insoluble in 50% (v/v) ethanol (*11*, *12*).

Several methods for lentinan and/or β-glucans quantitation from edible mushrooms have been reported. Minato et al. (*11*) and Mizono et al. (*12*) reported the development and use of an enzyme-linked immunosorbent assay to detect levels of lentinan in mushrooms. Widespread adoption of the procedure of Mizono et al. (*12*) has not occurred, possibly because of the limited availability of the lentinan reactive antibody. Manzi and Pizzoferrato (*13*) adapted the protocol of McCleary and Glennie-Holmes (*14*), by which the β-glucans from barley and malt are first digested to glucose by a highly purified β-glucanase (lichenase) from *Bracillus subtilis* and β-D-glucosidase, and then glucose is quantified by changes in absorbance after oxidization by glucose oxidase and peroxidase. Brauer et al. (*15*) demonstrated that β-glucanase from *B. subtilis* and β-D-glucosidase did not degrade purified lentinan to glucose. Therefore, it seems unlikely that the glucans quantified by use of the Manzi and Pizzoferrato (*13*) method would include lentinan.

Brauer et al. (*16*) reported a method to quantitate a fraction that included lentinan. Aqueous extracts of shiitake mushrooms were fractionation by ethanol precipitation and size exclusion

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chromatography. Lentinan recovery during these two steps was quantitative (*16*). Total carbohydrate content of the partially purified fraction was determined colorimetrically after reaction with anthrone using the method of Brink et al. (*17*). The purity of the carbohydrate fractions after ethanol precipitation and gel chromatography was not established in the initial publication (*16*), and therefore they referred to the fraction as high molecular weight polysaccharides (HMWP). More recently, Brauer et al. (*15*) reported that >80% of the carbohydrates in the HMWP had the molecular weight reported for lentinan as determined by size exclusion chromatography. The relative proportions of carbohydrates eluting with a molecular weight of lentinan were similar among five shiitake strains differing in HMWP content (*15*, *16*). The HMWP fraction had only trace amounts of starch and/or glycogen (*15*).

Limited data comparing the effects of the two basic production systems, that is, log-grown and substrate-grown, on HMWP concentrations in shiitake mushroom have been reported (*16*). In general, HMWP concentrations were greater in log-grown than in substrate-grown shiitakes. Barros et al. (*18*) reported that the maturity stage of fruiting bodies from *Lactarius* sp. mushrooms affected the activity of antimicrobial activities. Therefore, there is a possibility that the developmental stage of the shiitake cap or fruiting body may determine the concentration of HMWP. The objectives of these studies were to determine the effects of shiitake strains, development stages of the mushroom caps, and duration of the saprophytic degradation of logs on the concentrations of HMWP in shiitake mushrooms.

MATERIALS AND METHODS

Log-Grown Shiitake Production Method. Spawn sources for this research were obtained from Field and Forest Products (Peshtigo, WI). Log-grown mushrooms were grown in Shirley, AR (approximately N 35.655, W 92.318). Inoculation (approximately 3 mL of volume) was accomplished using sawdust spawn inserted into 1.2 cm (diameter) × 2.5 cm (depth) holes drilled into logs in a diamond pattern configuration (15−22 cm × 4 −5 cm). Inoculated holes were then sealed with food-grade cheese-wax heated to approximately 190 °C. Logs averaged approximately 100 cm in length and 10−15 cm in diameter. Logs were cut green from white oak (*Quercus alba* L.) trees after leaf drop in the fall but before bud break in the spring (i.e., mid-November to late March).

The initial growing conditions involved outside incubation in a tight lean-to stack on a northern exposure and covered with cut eastern red cedar (*Juniperus virginiana* L.) boughs to provide approximately 90% shade. Lean-to stacks were maintained for approximately 9 months and hydrated periodically using Rainbird sprinklers (25PJDA-C impact sprinkler, San Diego CA). In the September and October immediately following inoculation, the initial fruiting began and logs were moved to "A-frame" stacks under a densely shaded, deciduous tree stand. Logs were left in this position to fruit (produce mushrooms) naturally outside. Fruiting usually occurred immediately after a heavy rainfall event at suitable ambient temperatures. Mushroom caps typically were collected at one of three development stages: bud, veil break, and open. Bud stage refers to the initial mushroom cap appearance. Buds are formed and visible when the cap is swollen and distinct from its stalk. Buds are usually dome-shaped between 1 and 2 cm in diameter. Veil break refers to the stage of mushroom cap development at which the veil begins to open and separate from the stalk, exposing the gills or lamellae. At veil break, the cap is still dome-shaped. The mushroom cap is flat and the outside edges are slightly curled, with the gills clearly exposed at the fully open stage. Over 3000 logs were inoculated for the studies described below. All experiments had three replications with each replication representing numerous logs. Three areas corresponding to the replicates were defined within the growth area. Logs representing a combination of inoculation dates, fungal strains, etc., were randomly assigned with the replication area. Logs within a replication area were rerandomized after each harvest.

Shiitake Strain Comparisons. This study compared the production of HMWP by 12 shiitake strains. Oak logs were inoculated between January 2002 and June 2004 (**Table 1**) with the intention of obtaining the first harvest of mushrooms in the spring of 2005. Inoculation date was based on the expected growth rate of the fungal strain. For instance, the cold weather strain CW-25 was expected to be slow growing; thus, logs inoculated in January 2002 were included in this study. Logs were inoculated in June 2004 with four strains (29-430, 569-430, MY-602, and NN-430) that were expected to have the fastest growth rates. Mushrooms were picked at veil break during the first harvest, which occurred between February 21 and May 17, 2005. Logs inoculated with cold weather strain 603 failed to yield enough caps for sampling and were not analyzed for HMWP. A second harvest occurred between September 5 and October 25, 2005. Logs inoculated with the cold weather strain CW-25 did not fruit until January 17, 2006. Mushrooms at the bud, veil break, and open developmental stages were collected.

Table 1. Summary of the Shiitake Strains Used To Produce Samples[a]

strain name	month of inoculation	fruiting for strain comparison	fruiting for cap development	fruiting for time after inoculation
Night Velvet	Jan-03	Mar-05; Oct-05	Oct-05	
Mori 290	Mar-03	Feb-05; Oct-05	Oct-05	
Biyang Flower	Apr-03	Apr-05; Sep-05	Sep-05	
Sefi 30	May-03	Mar-05; Oct-05	Oct-05	
K-6	Jan-04	Mar-05; Sep-05	Sep-05	
603	Jan-04	Sep-05	Sep-05	
762	Jan-04	Mar-05; Sep-05	Sep-05; Oct-06	
MY-602	Jun-04	Apr-05; Sep-05	Sep-05; Jun-06	
29-430	Jun-04	Apr-05; Oct-05	Oct-05; Oct-06	
CW-25	Jan-02	Feb-05; Jan-06	Jan-06	
569-430	Jun-04	Mar-05; Sep-05	Sep-05; Jun-06	Mar-08
569-430	Jan-06			Mar-08
569-430	Dec-06			Mar-08
NN-430	Jun-04	Mar-05; Oct-05	Oct-05; Oct-06	Apr-08
NN-430	Jan-06			Apr-08
NN-430	Dec-06			Apr-08

[a] Month and year (3 letter abbreviation for month, years between 2002 and 2008) refer to the time at which inoculations and harvests occurred.

Data from the first harvest (spring of 2005) and from veil break samples collected from the second harvest (fall of 2005) were analyzed to assess effects of shiitake strain on HMWP concentrations. The experimental design was shiitake strains being a main effect and harvests being a main effect and repeated measure. Analysis of variance and least-squares means and standard errors of the means (LSSE) were calculated using PROC MIXED of SAS (*19*). Degrees of freedom (DF) in the nominator for the *F* values were 1 and 10 for the main effects of harvests and strains, respectively. DF for the denominator of the *F* value were 44.

Mushroom Development/Maturation Studies. Mushrooms representing bud, veil break, and open developmental stages were collected between September 2005 and January 2006 (**Table 1**) from oak logs inoculated with 1 of the 12 strains. Mushrooms representing bud, veil break, and open developmental stages were also collected from the third harvest from logs inoculated with 1 of 5 shiitake strains (**Table 1**). Fruiting of this third harvest began on June 6, 2006, for logs inoculated with strains 569-430 and MY-602. The other three strains (NN-430, 29-430, and 603) fruited between October 5, 2006, and November 24, 2006.

Data from the second harvest of logs inoculated with 1 of the 12 shiitake strains were analyzed statistically using a completely randomized block design with strains and developmental stage as main effects using PROC GLM of SAS (*19*). Least-squares means and LSSE were computed by PROC GLM. Data from the five strains that were measured in the second and third harvests were statistically analyzed using the two harvests as repeated measures and strains and cap development stage as main effects. Analysis of variance of repeated measures was conducted using PROC MIXED of SAS (*19*). DF for the main effects of harvests, shiitake strains, and stages of mushroom development were 1, 4, and 2, respectively. DF for the error term were 68. Least-squares means and LSSE were computed by PROC MIXED.

Length of Saprophytic Association Study. Oak logs were inoculated with one of the two shiitake strains: 569-430, a strain fruiting under wide range of temperatures, and NN-430, a strain fruiting mainly during cold temperatures (< 5 °C). These two strains were chosen because mushrooms from these strains tended to have higher HMWP concentrations. Logs were inoculated in June 2004, between January and March 2006, and in December 2006. Inoculated logs were allowed to fruit in response to environmental conditions postinoculation. Thus, at the beginning of 2008, logs from these three inoculations represented a range of saprophytic degradation. Fruiting from all of the NN-430 inoculations started after February 15, 2008, and was completed by March 20, 2008. Due to unusual weather conditions (cool and wet) during the spring of 2008, a forced fruiting of logs inoculated with strain 569-430 was necessary to obtain samples. A subset of logs was randomly selected from each inoculation of the 569-430 strain. These logs were submerged in water for 48 h. Fruiting started 3−5 days after soaking, following incubation at 18 and 21 °C at 90% relative humidity during the first week in April 2008 (**Table 1**). Only mushroom caps in the bud stage were harvested. Data were analyzed as a completely randomized block design with strains and time after inoculation as main effects using PROC GLM (*19*). Least-squares means and LSSE were used for mean comparison when *F* values indicated a significant effect.

Sample Processing and HMWP Analyses. Collected mushroom caps were sliced (approximately 5 mm thick) and then dried at room temperature (20−22 °C) with circulating air in an industrial type food dryer (professional model FD-108, MarVlizer, Madison WI) immediately after harvesting. Dried samples were ground to a 20-mesh powder using a grinding mill (model 4-E, Straub, Hatboro PA) and stored at −20 °C until analyses for polysaccharides. All samples from a harvest period were collected and prepared prior to analyses. The HMWP content was determined as described previously (*15*, *16*). Briefly, HMWP in finely ground mushrooms were extracted in hot water overnight. The HMWP fraction was prepared by participation with 50% (v/v) ethanol and size exclusion chromatography, and total carbohydrate analysis was performed on the resulting fraction. Duplicate determinations were routinely performed on each sample. Additional replicates of analyses were performed until a coefficient of variation among analyses for a sample's HMWP concentration was < 5%.

RESULTS

Shiitake Strain Comparisons. Both shiitake strains and harvests affected HMWP concentrations of mushrooms collected during the spring and fall of 2005. The *F* values for the effect of harvests and shiitake strains were 404.4 ($P < 0.001$) and 20.57 ($P < 0.001$), respectively. The *F* value for the interaction between harvests and strains was 5.27 ($P < 0.001$). HMWP concentration of mushrooms from the first harvest in the spring of 2005 greatly exceeded that of the mushrooms from the fall 2005 harvest. Means across strains were 16.7 and 5.5 mg of HMWP g^{-1} for the first and second harvests, respectively. It is not known if the difference between harvests represented an effect of length of saprophytic degradation or of differences in sample processing. Results from an experiment to assess the effects of length of saprophytic association on HMWP concentrations are described later.

Mushrooms from the shiitake strain Night Velvet tended to have the lowest concentrations of HMWP (**Table 2**). Mushrooms from strain NN-430 had the highest HMWP concentrations in both harvests (**Table 2**).

Shiitake Mushroom Development/Maturation Studies. Concentrations of HMWP were significantly affected by shiitake strain and developmental stage (**Table 3**). There was a highly significant ($P < 0.001$) interaction between shiitake strain and developmental stage. When harvested at bud break, HMWP concentrations in mushrooms varied among shiitake strains from a high of 12.3 mg g^{-1} for strain NN-430 to a low of 1.5 mg g^{-1} for Night Velvet. Mushrooms harvested at veil break and fully open stages had similar ranges in HMWP concentrations across shiitake strains (data not shown).

Table 2. Effects of Shiitake Strain on Concentrations of HMWP in Mushrooms from Harvests in the Spring and Fall of 2005[a]

	HMWP (mg g^{-1})	
strain name	spring 2005 harvest	fall 2005 harvest
Night Velvet	5.8	1.9
MY-602	11.4	2.7
SEFI 30	16.5	6.8
K-6	18.1	4.5
762	20.5	3.8
569-430	21.5	7.5
M-290	11.2	4.3
BYF	13.0	4.4
CW-25	18.1	5.7
29-430	19.2	8.0
NN-430	28.7	10.4

[a] Mushrooms were collected in the veil break stage. These harvests represent the first and second harvests, respectively. Data are the least-squares means across replications with LSSE of 1.31 mg g^{-1}.

Table 3. Analysis of Variance Summarizing the Effect of Shiitake Strain and Mushroom Development Stage on the Concentrations of HMWP in Mushrooms Harvested in the Fall of 2005 (Second Harvest)[a]

		HMWP concentration	
source of error	DF	MSS	*F* value[b]
strain (S)	11	43.59	33.63***
developmental stage (D)	2	31.50	24.30***
S × D	22	11.17	8.62***
error	72	1.30	

[a] Abbreviations: DF, degrees of freedom; MSS, mean sum of squares. [b] ** and *** denote that the *F* value was significant at $P < 0.01$ and 0.001, respectively.

When data were averaged over the 12 shiitake strains, increasing mushroom maturation was associated with decreases in HMWP content. Content of HMWP declined from a high in bud break mushrooms (mean of 6.1 mg g^{-1}, LSSE = 0.19) to 5.4 and 4.3 mg g^{-1} for mushrooms harvested at veil break and fully open, respectively.

The significant interactions between shiitake strain and mushroom developmental stage indicated that not all of the shiitake strains followed the same trend for changes in HMWP concentrations with cap maturation. Seven of the 12 strains (Night Velvet, SEFI 30, 762, 569-430, MY-602, NN-430, and CW-25) expressed the general pattern of decreasing HMWP concentrations with increasing mushroom maturation. The decline in HMWP with maturity varied considerably among these seven strains, with Night Velvet and SEFI 30 representing the extremes. The HMWP concentrations of Night Velvet mushrooms decreased approximately 75% from bud break to fully open (from 4.7 to 1.5 mg of HMWP g^{-1}) compared to only a 20% decrease with SEFI 30 (from 7.2 to 5.7 mg of HMWP g^{-1}). Mushrooms from three strains (BYF, K-6, and 29-430) varied little in HMWP concentrations with mushroom cap maturity. The HMWP concentrations of mushrooms from two strains (M-290 and 603) increased slightly with maturation, although the means for the two strains at the three developmental stages were not significantly different.

In the experiment in which five strains and two harvests were variables, HMWP concentrations were significantly ($P < 0.001$) affected by harvest, shiitake strain, and development stage of the mushroom. The interaction between strain and developmental stage was also significant ($P < 0.05$). The overall means for HMWP concentrations in the third harvest were significantly lower than for the second harvest, 5.8 versus 3.8 mg of HMWP

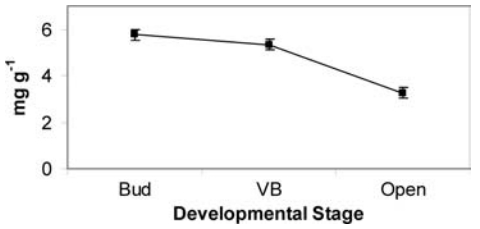

Figure 1. Effects of mushroom developmental stage on concentrations of HMWP. In the figure, developmental stages bud break, veil break, and fully open are abbreviated Bud, VB, and Open, respectively. Data are means across five shiitake strains and two successive harvests in the fall of 2005 and spring of 2006. Bars denote LSSE, which equals 0.2 mg g^{-1}.

Table 4. Analysis of Variance Summarizing the Effect of Length of Saprophytic Association and Shiitake Strain on Concentrations of HMWP in Mushrooms Harvested in the Bud Break Stage in the Spring of 2008[a]

		HMWP content	
source of error	DF	MSS	*F* value[b]
length of association (A)	2	11.27	32.01***
shiitake strain (S)	1	247.16	701.72***
A × S	2	4.37	12.42***
error	12	0.35	

[a] Abbreviations: DF, degrees of freedom; MSS, mean sum of squares. [b] *** denotes that the *F* value was significant at $P < 0.001$.

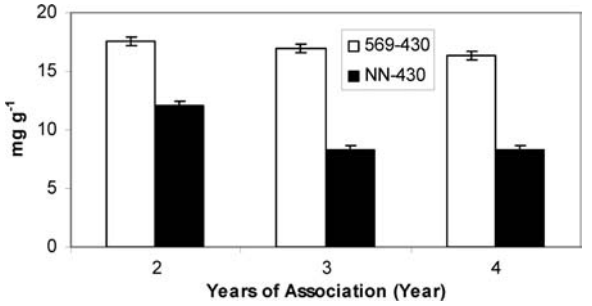

Figure 2. Effects of length of saprophytic association on the concentrations of HMWP in mushrooms from two shiitake strains harvested in the bud break stage. Data from 569-430 and NN-430 are presented by white and black bars, respectively. Bars representing the LSSE are presented. Mushrooms were harvested in the spring of 2008 from logs inoculated in June 2004, January 2006, or December 2006, resulting in lengths of saprophytic associations of 2, 3, or 4 years, respectively.

g^{-1}. It is not known if this decrease reflects the effects of either the length of saprophytic association or slight differences in sample processing because samples from each fruiting were processed separately. A direct comparison to assess the effects of length of saprophytic association is described below.

The effects of mushroom development on HMWP concentrations were similar to those observed in the earlier study (**Figure 1**). Concentrations of HMWP decreased with maturation of the mushroom cap, with the greatest decrease occurring between veil break and fully open.

Length of Saprophytic Association Study. The concentrations of HMWP in mushrooms collected at the bud break developmental stage in the spring of 2008 were significantly affected by the length of the saprophytic association and the shiitake strain used to inoculate the logs (**Table 4**). There also was a significant interaction between the length of the association and shiitake strain on HMWP concentrations. Mushrooms from strain 569-430 had higher levels of HMWP than NN-430, when averaged across time after inoculation, 17.0 versus 9.6 mg g^{-1} (LSSE of 0.2).

Concentrations of HMWP decreased with increasing age of the saprophytic association, when concentrations were averaged across shiitake strains. However, the magnitude of the decrease in HMWP concentrations with the length of the saprophytic association differed between the two shiitake strains (**Figure 2**). With bud break mushrooms of strain 569-430, HMWP concentrations declined progressively as the age of the inoculated logs increased from 2 to 4 years, whereas there was large decrease between 2 and 3 years with bud break mushrooms of strain NN-430. Differences in the rates of decline of HMWP concentrations among the two strains were reflected in the significant *F* values for the length of association by shiitake strains in the analysis of variance.

DISCUSSION

The results from this study indicate that the concentrations of HMWP in shiitake mushrooms are influenced to a large degree by the fungal strain phenotype and environmental characteristics that a producer can manage. Concentrations of HMWP among shiitake strains varied as much as 8-fold from lowest to highest levels during a fruiting. Despite complex genetic and environment interactions on HMWP concentrations, some generalizations were revealed: (1) concentrations of HMWP decreased as shiitake mushroom caps matured and (2) HMWP concentrations decreased as the length of the saprophytic association increased.

Recommendations for producers of log-grown shiitake interested in producing mushrooms high in HMWP are difficult because of the interaction of various treatments with shiitake strains. However, the following management protocol is recommended to producers targeting the fresh mushroom market with a product high in HMWP: (1) inoculate oak logs with either shiitake strain NN-430 or 569-430, and (2) harvest mushrooms in the bud to veil break stage for the first year of fruiting. Mushrooms from shiitake strains NN-430 and 569-430 consistently had higher concentrations of HMWP than the other 12 strains reported in this study. An advantage of inoculating some logs with 569-430 and others with NN-430 would be a longer availability of mushrooms with high HMWP. Strain 569-430 is more likely to produce mushrooms in the spring and fall, whereas strain NN-430 is more likely to produce mushrooms in the winter, at least under climate conditions similar to the Ozark region of Arkansas (data not shown). A similar management protocol would be advisible for producers growing mushrooms from which HMWP would be extracted. For such a marketing plan, logs inoculated with shiitake strains NN-430 and 569-430 may be superior to alternative strains.

ABBREVIATIONS USED

DF, degrees of freedom; HMWP, high molecular weight polysaccharides; LSSE, least-squares standard error; MSS, mean sum of squares.

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