Autolysis of Lentinan, an Antitumor Polysaccharide, during Storage of *Lentinus edodes*, Shiitake Mushroom

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The lentinan contents in the *Lentinus edodes* fruit body during storage were examined by ELISA method using anti-lentinan antibodies. The lentinan content (12.8 mg·g^{-1} dw) before storage decreased to 3.7 mg·g^{-1} dw over 7 days at 20 °C. However, it only slightly decreased at 1 °C and only decreased to 9.3 mg·g^{-1} dw at 5 °C. Glucanase activity, which seems to be associated with lentinan degradation, increased more during storage of *L. edodes* at 20 °C than it did at lower temperatures. In addition, only glucose was detected as a degraded product from lentinan by the glucanase. This suggested that this enzyme would fit the profile of an *exo*-type glucanase. Also, polyphenol oxidase activity, known as an index of freshness reduction in the mushroom, increased ~2.7-fold (to 61.5 units·mg^{-1}) over 7 days during storage at 20 °C. However, its activity changed little during storage at lower temperatures. These results indicate that the reduction during storage of the quality of *L. edodes* as a functional food is accompanied by the decrease of lentinan, and by browning, and that *exo*-glucanase plays an important role in the decrease of lentinan content.

Keywords: *Lentinan; antitumor polysaccharide; Lentinus edodes; polyphenol oxidase; exo-glucanase; ELISA*

INTRODUCTION

It is well-known that the *Lentinus edodes*, "shiitake mushroom", fruit body is a functional food, effective on various tumors. Chihara et al. (1969, 1970) isolated and purified lentinan, a polysaccharide possessing significant antitumor activity, from hot water extracts of *L. edodes* and also reported that lentinan content was 15.5 mg in 100 g of fresh fruit bodies of *L. edodes*. The structure of lentinan was reported as β-1,3-linked-D-glucan with β-1,6 branches (Chihara et al., 1970; Sasaki and Takasuka, 1976; Saito et al., 1977, 1979). Several investigators have reported that lentinan showed strong host-mediate antitumor activities against various tumors, via so-called activations of T-cells, NK cells, and macrophages (Maeda and Chihara, 1973; Hamuro et al., 1980; Freunhauf et al., 1982; Gergely et al., 1988; Ladányi et al., 1993).

L. edodes is one of the most common edible mushrooms in Japan. Minamide et al. (1980) investigated the maintenance of quality during storage. Their results indicated that quality reduction of the mushroom is closely related to its browning. However, the relationship between quality reduction and the decrease of lentinan content in the mushroom has not yet been reported. It has remained obscure whether lentinan contents change during storage, in part due to the difficulty of isolating and identifying lentinan from the mushroom. In this paper we report the change in lentinan content of *L. edodes* during storage by ELISA, using anti-lentinan antibodies prepared previously (Mizuno et al., 1996). Furthermore, we attempted to clarify a relationship between lentinan degradation and glucanase activity during storage of *L. edodes*. In addition, polyphenol oxidase (PPO) activity, which reflects the browning of mushrooms, was measured as an index of quality in mushrooms.

MATERIALS AND METHODS

Materials. *L. edodes* (Berk.) Sing, commonly called "shiitake", cultivated on sawdust-based cultures in Yachiyo, Hyogo, Japan, was used. Immediately after harvesting, the fruiting body portions of five mushrooms were packed in pored polyethylene film bags (350 × 400 mm, 0.03 mm thickness) and stored at 1, 5, and 20 °C, respectively. After storage for 1, 3, 5, and 7 days, each sample was sliced, frozen in liquid nitrogen, and kept at −80 °C prior to analysis.

Determination of Lentinan Contents. Antibodies against the lentinan from *L. edodes* were prepared according to the method described previously (Mizuno et al., 1996). Purified lentinan was kindly supplied by Ajinomoto Co. (Japan). A crude lentinan fraction from the sample was prepared essentially according to the method of Chihara et al. (1970). The fresh *L. edodes* fruit body was homogenized with liquid nitrogen by a Waring blender and lyophilized. The lyophilized powder sample (10 g) was extracted with 100 mL of hot water for 10 h, and then the suspension was filtered to remove insoluble matter. The crude lentinan fraction was obtained by precipitation with an equal volume of ethanol to filtrate. The precipitate was centrifuged and lyophilized. The lentinan contents in the crude lentinan fractions were determined by inhibition assay of ELISA.

Assay of Polyphenol Oxidase Activity. PPO activity was determined according to the spectrophotometric method using chlorogenic acid as the substrate (Gong et al., 1993). The fruit body of *L. edodes* was homogenized in a Waring blender with 10-fold 0.05 M phosphate buffer (pH 6.0). The suspension was centrifuged at 10000*g* for 10 min at 4 °C. The supernatant was fractionated by addition of solid $(NH_4)_2SO_4$ to 80% saturation. The precipitate was collected by centrifugation (15000*g*, 10 min), redissolved in the same phosphate buffer solution (pH 6.0), and centrifuged again to remove insoluble materials. The obtained supernatant was desalted in the

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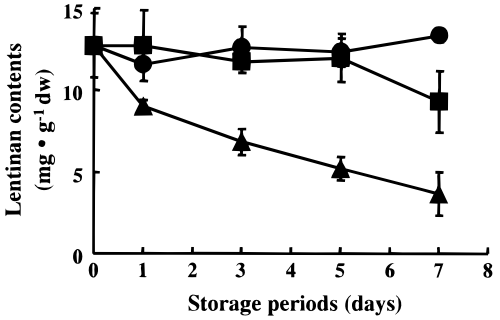

Figure 1. Changes in lentinan contents of *L. edode*s during storage. *L. edodes* fruit bodies were stored at 1 °C (●), 5 °C (■), and 20 °C (▲). All data points are mean ± SD ($n = 3$).

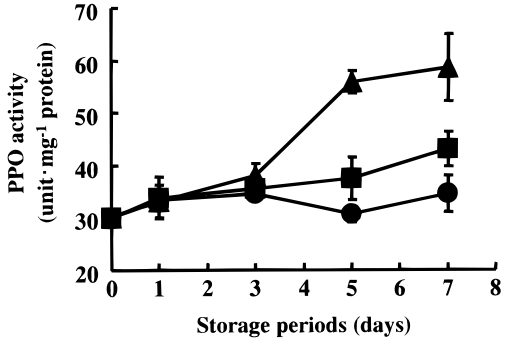

Figure 2. Changes in PPO activity of *L. edode*s during storage. Symbols are the same as in Figure 1. All data points are mean ± SD ($n = 3$).

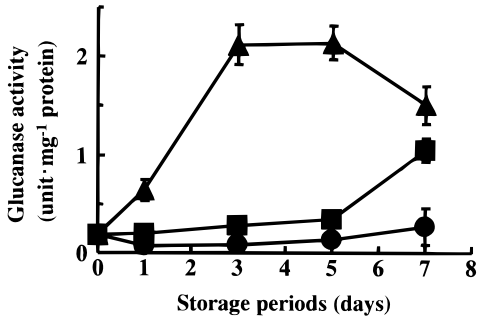

Figure 3. Changes in glucanase activity of *L. edode*s during storage. Symbols are the same as in Figure 1. All data points are mean ± SD ($n = 3$).

phosphate buffer solution using a PD-10 column (1.6 × 5 cm, Pharmacia LKB) and then used as an enzyme solution. The reaction solution, consisting of 1.0 mL of McIlvaine buffer (pH 4.5) with 3.5 mg·mL^{-1} substrate and 40 μL of enzyme solution, was incubated at 24 °C for 5 min. A change of absorbance of 0.01 per minute at 420 nm was defined as 1 unit of enzyme activity.

Assay of Glucanase Activity. All operations for the preparation of enzyme extracts were performed according to the procedure described by Ohga (1992). The fruit body of *L. edodes* was added to a cold, 10-fold 10 mM acetate−acetic acid buffer solution (pH 4.2) and homogenized in a Waring blender. The suspension was centrifuged at 10000*g* for 10 min. The supernatant was then fractionated by addition of solid $(NH_4)_2SO_4$ at pH 4.2 to 80% saturation. After 1 h, the precipitate was collected by centrifugation at 15000*g* for 20 min, redissolved in a 1 mM acetate−acetic acid buffer solution (pH 4.2), and then centrifuged to removed insoluble material at 15000*g*. The crude enzyme solution was desalted through a PD-10 column equilibrated with a 1 mM acetate−acetic acid buffer and stored at −80 °C prior to assay. The glucanase activity was determined using lentinan isolated from the *L. edodes* fruit body as substrate. Assay was performed in a 30 mM acetate−acetic acid buffer solution (pH 4.2) with 0.2 mg·mL^{-1} substrate at 40 °C for 30 min, and the amount of the released reducing sugar was determined by using the Somogyi−Nelson method. One unit of activity was defined as 1 μmol of reducing sugar released (as glucose equivalents) per minute.

Isolation and Identification of the Degradation Product from Lentinan. The degradation product of lentinan digested by the glucanase was analyzed by gel filtration chromatography. The crude enzyme solution (10 units·mL^{-1}, 1 mL) prepared as previously described was added to 10 mL of a 1 mg·mL^{-1} lentinan solution (pH 4.2) and then incubated for 15 or 30 min at 40 °C. After the enzymes had been inactived by heating for 10 min at 100 °C, the incubation mixture was applied to a Sephacryl S-300 HR column (Pharmacia Biotech, 1.6 × 90 cm) and eluted with a phosphate buffer solution (pH 7.2, 3 mL fractions). Glucose contents in the fractions were monitored according to the anthrone−H_2SO_4 method at 620 nm using glucose as the standard sugar. The fraction corresponding to $<3 \times 10^3$ Da was collected and further identified by HPLC on an Asahipak NH2P50 column (Shodex, Japan) using a 70% aqueous CH_3CN solution as an effluent at a flow rate of 1.0 mL·min^{-1} at 30 °C. The detector was an L-3300 RI detector (Hitachi, Japan).

RESULTS AND DISCUSSION

Decrease in the Lentinan Contents of *L. edodes* during Storage. Figure 1 shows the time course change in lentinan contents in a hot water extract from *L. edodes* during storage at 1, 5, and 20 °C. The *L. edodes* fruit body contained 12.8 mg·g^{-1} dw of lentinan immediately after harvesting. It was seen that the change in lentinan content during storage was markedly different in relation to the storage temperature. The amounts of lentinan in *L. edodes* rapidly decreased during storage at 20 °C and reached 3.7 mg·g^{-1} dw after 7 days. Although the initial level of lentinan was maintained during storage for 5 days at 5 °C and for 7 days at 1 °C, the amount of lentinan in *L. edodes* decreased to 9.3 mg·g^{-1} dw after 7 days at 5 °C. These results indicated that the lentinan content in the fruit body decreased rapidly during storage at 20 °C and that low-temperature storage was effective to maintain lentinan contents in *L. edodes*. Moreover, PPO activity was also examined as an index of the reduction of quality during storage of *L. edodes* (Figure 2). It has been reported that the browning of the fruit body is caused by the increased PPO activity during storage (Minamide et al., 1980a−c; Yamaguchi et al., 1988). As shown in Figure 2, PPO activity increased to 58.6 units·mg^{-1} at 20 °C during storage of *L. edodes*. However, PPO activities changed little during storage at lower temperatures. This result was consistent with the reports of Minamide et al. (1980a,b) and Gong et al. (1993) concerning the maintenance of freshness of *L. edodes* during storage.

Change in Glucanase Activity during Storage of the Mushroom *L. edodes*. The changes in glucanase activity during storage of *L. edodes* were measured by the Gong method using lentinan as a substrate to make clear the degradation mechanism of lentinan. The glucanase activity was at a very low level (0.27 unit·mg^{-1}) immediately after the mushrooms had been harvested, and it was only slightly enhanced during storage for 5 days at low temperatures (1 and 5 °C). The activity rose to 1.05 units·mg^{-1} in a storage at 5 °C after 7 days, but no significant changes were observed during storage at 1 °C. On the other hand, in a storage at 20 °C, glucanase activity rapidly increased and peaked to 2.12 units·mg^{-1} after 3 days (Figure 3). These results suggest that the decrease in lentinan content during storage of *L. edodes*

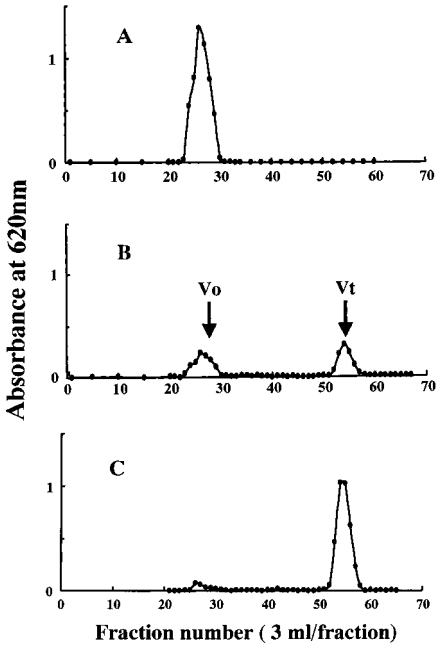

Figure 4. Gel filtration pattern on Sephacryl S-300 HR of the enzymatic digest of lentinan by lentinan hydrolase from *L. edodes*: (A) gel filtration pattern of lentinan; (B) after incubation for 15 min; (C) after incubation for 30 min. A solution of lentinan with an enzyme extract was incubated in a 30 mM acetate buffer, pH 4.2, at 40 °C. The hydrolysate was applied to a Sephacryl S-300 HR column (1.6 × 90 cm) by filtration with $^1/_{15}$ M phosphate buffer, pH 7.2, at 22 mL·h^{-1}. Each fraction was assayed for total sugar content according to the anthrone−H_2SO_4 method. The elution positions of blue dextran (V_0: $M_w > 4 \times 10^5$) and glucose (V_t: $M_w < 2 \times 10^3$) are shown.

might be correlated closely to the increase in glucanase activity (Figures 1 and 3). Thus, it is inferred that this glucanase plays an important role in the degradation of lentinan during storage of the mushroom, particularly when the storage temperature is as high as 20 °C.

Mode of Action of *L. edodes* Glucanase on Lentinan. Isolation and identification of the enzymatic degradation products were attempted to clarify the mode of action of *L. edodes* glucanase on lentinan. Elution patterns of the degradation products on Sephacryl S-300 HR gel are shown in Figure 4. On the elution pattern of the mixture incubated for 15 min (Figure 4B), two peaks from lentinan were detected, and the latter product was eluted at a range of $<3 \times 10^3$ Da. After 30 min of incubation, the peak corresponding to lentinan had nearly disappeared, and another peak was enhanced (Figure 4C). These facts suggested that the lentinan was digested to a product $<3 \times 10^3$ Da almost within 30 min. To identify this degradation product, it was further subjected to HPLC analysis. A single peak on the chromatogram was detected at a retention time of 6.45 min, and it coincided with that of authentic glucose. Also, the negative-SIMS mass spectrum of the peak agreed with that of glucose (data not shown). The results suggested that the degradation product was identical with glucose. Moreover, no oligomeric sugars could be detected in a degradation mixture digested for 15 and 30 min. These results assumed that this enzyme might be an *exo*-type glucanase.

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