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An exo β-1,3-glucanase synthesized de novo degrades lentinan during storage of *Lentinule edodes* and diminishes immunomodulating activity of the mushroom

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Abstract

Two β-1,3-glucanase isoenzymes, GNase I and II, were isolated from *Lentinule edodes* fruiting body. They degraded lentinan in an exo manner during storage of the mushroom. GNase I and II were monomeric proteins with apparent molecular masses of 72.9 and 74.7 kDa, respectively. It was shown that K_m values for GNase I and GNase II against lentinan were 1.02 and 2.41 μM, respectively. Moreover, it was demonstrated by western blotting analysis that this enzyme was synthesized de novo in *L. edodes* during storage at 20 °C, but not at 1 or 5 °C. And this pattern of western blot was consistent with that of glucanase activity during storage of *L. edodes*. In addition, the immunomodulating effects of lentinan on TNF-α and NO production were diminished with its degradation by this enzyme. These results suggested that synthesis of exo β-1,3-glucanases were induced under storage conditions at 20 °C, and lentinan degradation by their attack might result in the depletion of immunomodulating activity of *L. edodes*.

Keywords: Lentinan; Antitumor polysaccharide; Exo β-1,3-glucanase; De novo synthesis; Immunomodulating activity

1. Introduction

It is well known that lentinan has been isolated as an antitumor β-glucan, and that it can be purified from a hot water extract of *Lentinule edodes* fruiting bodies (Chihara, Maeda, Hamuro, Sasaki, & Fukuoka, 1969; Chihara, Hamuro, Maeda, Arai, & Fukuoka, 1970). The structure of lentinan was reported as β-1,3-linked-D-glucan with β-1,6 branches (Chihara et al., 1969; Sasaki & Takasuka, 1976; Saito, Ohki, Takasuka, & Sasaki, 1977; Saito, Ohki, & Sasaki, 1979). We have already reported that the amount of lentinan decreased during storage of *L. edodes* at 20 °C, and that β-glucanase activity increased with the decrease in lentinan (Minato, Mizuno, Terai, & Tsuchida, 1999a). This finding was suggested to be caused by the enzymatic degradation of lentinan by β-1,3-, and/or β-1,6-glucanase activity during storage of *L. edodes*. In this study, to elucidate how these enzymes degrade lentinan in *L. edodes* during storage, we isolated and purified the β-glucanases, that correlated with lentinan degradation, from *L. edodes*, and their properties and pattern of synthesis during storage of the mushroom were examined.

Lentinan is also known as a type of biological response modifier (Hamuro & Chihara, 1984). Its antitumor action is considered host-mediated, since this polysaccharide does not show any direct cytotoxicity against tumor cells (Maeda & Chihara, 1973). It is thought that lentinan augments the immune response through the modulation of the function of phagocytes such as macrophages (Maeda, Hamuro, & Chihara, 1971; Chihara, Suga, & Hamuro, 1987). It has been reported that lentinan possessed an immunomodulating effect on the activation of a variety of macrophage functions (e.g. interleukin-1 and superoxide anion

Abbreviations: CM, carboxymethyl; ELISA, enzyme linked immunosorbent assay; GNase, β-1,3-glucanase; NO, nitric oxide; PMSF, phenylmethanesulphonyl fluoride; TNF-α, tumor necrosis factor-α.

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production, phagocytosis, and cytotoxicity) (Freunhauf, Bonnard, & Heberman, 1982; Ábel, Szöllõsi, Chihara, & Fachet, 1989; Herlyn, Kaneko, Rewe, Aoki, & Koprouski, 1983; Nanba, Mori, Toyomasu, & Kuroda, 1987; Ladányi, Tímár, & Lapis, 1993). Recently, Kerékgyártó, Virag, Tanko, and Chihara (1996) have reported that murine macrophages stimulated with lentinan produced tumor necrosis factor (TNF)-α. TNF-α is recognized as the primary cytokine produced, for the most part, by activated macrophages; it is an important host defense molecule that affects tumor cells (Carswell et al., 1975). Moreover, much attention has been focused on the role of nitric oxide (NO), which has shown itself to be an essential mediator of diverse functions, including immunoresponse, vasodilatation, neurotransmission, inhibition of platelet aggregation, and inflammation (Lowenstein & Snyder, 1992). NO was first recognized in mammalian physiology as a mediator of macrophage actions. Reactive nitrogen intermediates are thought to play a significant role in tumoricidal and microbicidal activities. This effect may be due to an increase of preferential formation of peroxynitrite, which can be formed by a direct reaction of NO with the superoxide radical. It has been reported that murine macrophages stimulated with lentinan released NO (Irinoda, Masihi, Chihara, Kaneko, & Katori, 1992). Thus, it seems that enhancement of TNF-α and NO production through macrophages may actually be a sign of the antitumor activity of lentinan. We have also reported that the immunomodulating activity of *L. edodes* in TNF-α and NO production depleted as decrease in lentinan contents during storage at 20 °C (Minato et al., 1999b). In this study, moreover, we investigated the changes in TNF-α and NO production from murine peritoneal macrophages that were stimulated with lentinan degradation products by the purified β-glucanase. This aim was to clarify whether or not the purified glucanase from the *L. edodes* fruiting body influenced the immunomodulating effects of lentinan.

2. Materials and methods

2.1. Materials

Lentinan was isolated from *L. edodes* fruiting bodies according to the method designated by Chihara et al. (1970). Laminarin, β-1,3-glucan, from *Laminaria digitata* was purchased from Sigma Chemical Co. (St Louis, MO, USA). Pustulan from *Umbilicaria papullosa* was purchased from Calbiochem (San Diego, CA, USA). CM-Curdlan and CM-cellulose were purchased from Wako pure chemical industries, Ltd. (Osaka, Japan).

2.2. Purification of β-1,3-glucanase from L. edodes

All operations for the preparation of enzyme extracts were performed at 4 °C. *L. edodes* mushrooms (500 g) were stored for 4 days at 20 °C. The mushrooms were homogenized in an extraction buffer (1 l), which consisted of 10 mM sodium acetate buffer (pH 4.2) containing 10 mM EDTA, 1 mM PMSF, and 20 mM β-mercaptoethanol. After the homogenate was formed, the insoluble material was removed by centrifugation (10,000*g*, 30 min), and the supernatant, a crude enzyme, was precipitated by salting-out with 80% saturation of $(NH_4)_2SO_4$. The precipitant was redissolved in the extraction buffer, and dialyzed against a 1 mM sodium acetate buffer (pH 4.2) for 24 h. Extracts were applied to a DEAE Sepharose CL-6B column (5 cm × 80 cm, Amersham Pharmacia Biotech, Amersham, UK) that had been equilibrated in 20 mM sodium acetate buffer (pH 6.0). After the elution of unbound proteins at a flow rate of 20 ml min^{-1}, bound proteins were eluted with 0.5 M NaCl in the same buffer (pH 6.0). The fractions which demonstrated β-glucanase activity were collected and fractionated with 80% $(NH_4)_2SO_4$. Protein precipitates were redissolved in the extraction buffer, and then dialyzed against the 1 mM sodium acetate buffer. The extracts were stored at − 80 °C prior to further use as a crude enzyme.

All chromatographic procedures were performed using an FPLC system and pre-packed columns (Amersham Pharmacia Biotech). For the anion-exchange chromatography, a Mono-Q column HR 5/5 (5 mm × 50 mm) equilibrated with 20 mM sodium acetate buffer (pH 6.0) was used. A prepared crude enzyme extract was filtered through DISMIC-13cp (0.2 μm-pore-size) filters (ADVANTEC, Japan) and applied to the column in the equilibration buffer. Absorbed protein was eluted with a linear NaCl gradient (0–0.2 M) in the same buffer at low rate of 1 ml min^{-1}; and 1 ml fractions were then collected. Active fractions were further purified by FPLC gel permeation chromatography with a Superose 12 column HR 10/30 (10 mm × 300 mm) equilibrated with 50 mM sodium acetate buffer (pH 5.0) containing 150 mM NaCl. The sample was applied and eluted with the equilibration buffer at a flow rate of 0.4 ml min^{-1} and collected in 0.4 ml fractions. Molecular masses of the purified glucanases were estimated using aldolase (158 kDa), bovine serum albumin (BSA, 67 kDa), ovalbumin (43 kDa), and ribonuclease (13.7 kDa) as standards.

2.3. Gel electrophoresis

The homogeneity and the molecular masses of purified enzymes were determined by SDS–PAGE according to the method described by Laemmli (1970) using a 12.5% gel under reducing and non-reducing conditions. Molecular masses were determined with a rainbow marker (Amersham Pharmacia Biotech) containing myosin (220 kDa), phosphorylase b (97.4 kDa), BSA (66 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), and trypsin inhibitor (21.5 kDa) as standards. The gel was then stained with Coomassie Brilliant Blue R250.

2.4. Enzyme assay

β-Glucanase activity was routinely determined, in triplicate, using lentinan from *L. edodes* as the substrate. Assays were performed in 30 mM sodium acetate buffer (pH 4.2) with 0.1% (w/v) substrate at 40 °C for 30 min, and the amount of reducing sugar released was determined by the Somogyi–Nelson method (Nelson, 1944; Somogyi, 1952). One unit of enzyme activity was defined as 1 μmol of glucose equivalents released per minute. Protein was determined by the Coomassie Brilliant Blue reagent (Bradford, 1976) or Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951), using BSA as a standard.

2.5. Enzymatic properties and kinetics

Substrate specificities of the purified β-glucanases were determined against some β-D-glucans at 1.0 mg ml^{-1}. Activities against all substrates were determined reductometrically, as described above. Kinetic parameters were determined at 40 °C and at the respective optimum pH for each enzyme. All substrates were used over the concentration range 0.05–2.0 mg ml^{-1}, and then data were analyzed by curve fitting based on the Gauss–Newton method (McCormick & Salvadori, 1964).

2.6. Enzymatic digestion of lentinan

Lentinan was digested by the purified enzyme. The purified glucanase (100 units), prepared as already described, was added to 10 ml of 1 mg ml^{-1} lentinan solution in 30 mM sodium acetate buffer (pH 4.2) and then was incubated for 0.25 or 48 h at 40 °C. After inactivating the enzymes by heating them for 10 min at 100 °C, the incubation mixtures were applied to a Sephacryl S-300 HR column (1.6 × 90 cm^2, Amersham Pharmacia Biotech) and eluted with a phosphate buffer (pH 7.2, 3 ml fractions), in order to confirm the action modes of the purified β-glucanase isoenzymes. Using glucose as a standard, sugar contents in the fractions were monitored by the anthrone–H_2SO_4 method at 620 nm. The fraction corresponding to less than 3×10^3 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).

2.7. Preparation of anti-β-1,3-glucanase antibody

The β-1,3-glucanase (GNase I, 0.5 mg) was dissolved in 1 ml of phosphate buffered saline (PBS, pH 7.2). This solution was emulsified with an equal volume of Freund's complete adjuvant. The emulsion was injected subcutaneously at 10 different sites in the back of individual rabbits (New Zealand White, female, 16 weeks old). One-half volume of the β-1,3-glucanase in the first dosage was boosted again 2 weeks after the first injection. After the boost, the blood was collected to obtain anti-β-1,3-glucanase sera several times every week. The sera were stored at − 80 °C until use. After measurement of the titer of these antibodies, the rabbits were exsanguinated. Then, the IgG fractions of the antisera were isolated and purified by a Protein G column (0.5 × 10 cm^2, Amersham Pharmacia Biotech).

2.8. Western blotting analysis

Proteins were transferred to PVDF membranes (Amersham Pharmacia Biotech) under semi-dry transfer conditions after separation by SDS–PAGE. Blots were blocked in TPBS (0.05% Tween 20 in PBS) containing 20% skim milk overnight at 4 °C. Blots were washed with TPBS and then incubated for 1 h at room temperature with rabbit anti-*L. edodes* β-1,3-glucanase antibody IgG in TPBS containing 20% skim milk. Following incubation with the primary antisera, blots were washed four times with TPBS (5 min/wash). The blots were incubated with HRP-conjugated goat anti-rabbit IgG secondary antibody for 30 min at room temperature at a dilution of 1:2000. The blots were washed five times with TPBS. Detection of β-1,3-glucanase (GNase I) in *L. edodes* was performed by enhanced chemiluminescence according to the manufacturer's specifications (Amersham Pharmacia Biotech).

2.9. Preparation of peritoneal macrophages

Peritoneal macrophages were isolated from female BALB/c mice, 8 weeks old (Japan SLC, Shizuoka, Japan). The mice had been injected intraperitoneally with 2 ml of thioglycolate medium (DIFCO, Detroit, MI, USA) 3 days prior to peritoneal lavage with 10 ml of serum-free RPMI 1640 medium (GIBCO BRL, Grand Island, NY, USA). Collected cells were washed with RPMI 1640 and then plated in 24-well culture plates at a density of 1×10^6 cells per well. Cultures were incubated for 2 h at 37 °C and 5% CO_2 in order to cause adhesion to the plates. After incubation, the cultures were washed to remove all nonadherent cells. Fresh RPMI 1640 medium containing 10% fetal bovine serum (FBS) and various concentrations of samples were then added to the plates, and cultured for 24 h at 37 °C and 5% CO_2. After incubation, the supernatants of the cultures were collected and used for a nitrite assay; samples were then stored at − 80 °C until the TNF assay.

2.10. Measurement of the immunomodulating effects of lentinan degradation products on cytokine products

Lentinan was incubated for 0.25–48 h at 40 °C with purified enzyme solution prepared as substantial β-1,3-glucanase (100 units). After incubation at 100 °C for 10 min, the amount of lentinan in the incubation mixture was measured by an inhibition assay of ELISA as described

previously (Mizuno, Minato, & Tsuchida, 1996). The effect of the mixture on TNF-α and NO production from murine peritoneal macrophages was investigated.

TNF-α was measured by means of a cytolytic assay with actinomycin D-treated L929 cells (Takada, Ohno, & Yadomae, 1994); murine rTNF-α (Wako Ltd.) was used as the standard. L929 cells (2×10^5 cells ml^{-1}) were plated in 96-well culture plates in RPMI 1640 medium that included 5% FBS and cultured for 2 h. Fifty microliters of supernatant samples obtained from macrophages stimulated with extract of *L. edodes* or authentic lentinan and 50 μl samples of actinomycin D (4 μg ml^{-1}) were added to the culture plates, and then were cultured for 20 h at 37 °C and 5% CO_2. After incubation, the plates were washed and cell lysis was determined by staining with 0.1% crystal violet in ethanol/formaldehyde for 15 min at room temperature. After being washed with water and dried, the cells were dissolved in 100 μl of ethanol–PBS (1:1, vol/vol). The absorbance of the cell lysate in each well was measured by a microplate reader (MTP-120, Corona Electric Co. Ltd, Tokyo, Japan) at 570 nm (main) and 630 nm (reference).

NO, measured by the accumulation of nitrite as a stable end product, was determined by a microplate assay (Green et al., 1982). Briefly, 100 μl samples were incubated with an equal volume of Griess reagent (1% sulfanilamide/0.1% *N*-1-naphthylethylenediamine dihydrochloride/2.5% H_3PO_4) at room temperature for 10 min. The absorbance at 570 nm was determined with a microplate reader. Nitrite concentration was calculated by using sodium nitrite as a standard.

3. Results and discussion

3.1. Purification and properties of the β-1,3-glucanases from Lentinule edodes

In order to investigate the properties of β-glucanase that seem to influence the decrease in lentinan, β-glucanases were isolated and purified from *L. edodes* fruiting bodies. Two peaks that hydrolyzed lentinan were shown by the elution pattern on an anion-exchange (Mono-Q) column; these peaks were designated as GNase I eluted by a 99.0 mM of NaCl, and GNase II eluted by a 116.0 mM of NaCl, respectively (Fig. 1). These fractions with β-glucanase activity were further chromatographed respectively on a Superose 12 column. The elution patterns on the gel column of GNase I and II revealed single protein peaks that corresponded to fractions with showed β-glucanase activity (data not shown). Following SDS–PAGE under reducing and non-reducing conditions, both purified β-glucanases also exhibited single protein bands (Fig. 2). The molecular masses of GNase I and II were estimated to be 72.9 and 74.7 kDa, respectively. These results showed that the two purified glucanases were monomeric proteins. The N-terminal amino acid sequences of the two isoenzymes are

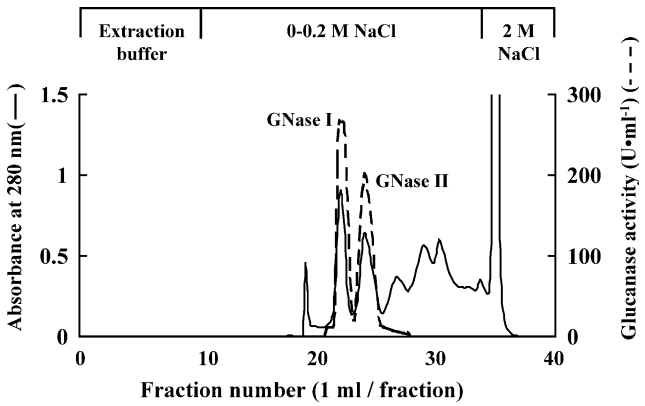

Fig. 1. Amino-exchange chromatogram of β-glucanases by FPLC. The fraction of *L. edodes* eluted with 0.5 M NaCl on DEAE-Sepharose CL-6B was applied on a Mono Q column. Fractions (0.4 ml) were assayed for protein (A_{280}) (—); and β-glucanase activity (…).

shown below:

GNase I	L	G	T	S	A	A	A	P	L	G
GNaseII	L	G	T	S	A	X	A	P	L	G

The substrate specificities of the *L. edodes* GNases have been compared using the linear (laminarin and pustulan), the substituted (CM-curdlan and CM-cellulose) and the side-branched (lentinan) β-glucans. Both β-glucanases hydrolyzed specifically β-1,6-branched β-1,3-glucans, lentinan from *L. edodes*, and laminarin from *L. digitata* (Table 1). Laminarin was hydrolyzed at the highest rate by GNase I and II (1.5- and 1.3-fold compared with lentinan, respectively), although lentinan was also hydrolyzed significantly by both glucanases. Therefore, the preferred substrate for the two isoenzymes was a laminarin, an essentially linear, soluble, β-1,3-D-glucan with a low degree of glycosyl substitution at *O*-6. In contrast, CM-curdlan (a substituted β-1,3-glucan) was degraded slightly by both β-glucanases (Table 1). GNase I and II were not effective at degrading

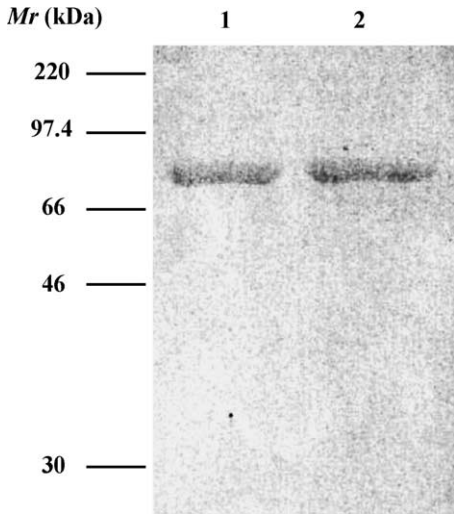

Fig. 2. SDS–PAGE of the purified *L. edodes* β-glucanases. Lane 1; purified GNase I, lane 2; GNase II.

Table 1
Relative rate of hydrolysis of β-glucan by β-glucanase purified from *L. edodes*

Substrate	Relative rate (%)	
	GNase I	GNase II
Lentinan	100	100
L. digitata laminarin	151	127
CM curdlan	1.8	0.5
Pustulan	No activity	No activity
CM cellulose	No activity	No activity

The relative rates of hydrolysis of the two β-glucanases against lentinan were arbitrarily set at 100% and correspond to 5.3 and 5.4 munits for GNase I and II, respectively.

pustulan (a β-1,6-glucan) or CM-cellulose (a substituted β-1,4-glucan). These results demonstrated that the both GNase I and II might specifically attack the β-1,3-linkages of the β-glucans. Therefore, both of the purified isoenzymes could be classified into the group of β-1,3-glucanase.

Moreover, the optimum pH of both β-1,3-glucanases were pH 4.2 (data not shown). A few fungal β-glucanases have been extensively characterized, a comparison of their physicochemical properties with those of the *L. edodes* GNases reveals some similarities. The molecular mass and optimum pH of each purified *L. edodes* GNase was similar and fell well within the range of values previously reported for other β-glucanases (Bielrcki & Galas, 1991; Stone & Clarke, 1992; de la Cruz, Pintor-Toro, Benítez, Llobell, & Romero, 1995; Pitson, Seviour, MaDougall, Woodward, & Stone, 1995).

The K_m values of the two purified *L. edodes* β-1,3-glucanases were determined by curve fitting over the concentration range of 0.05–2.0 mg ml^{-1} for lentinan, kinetic determinations were used (Table 2). The K_m value of GNase I for lentinan was lower than those of GNase II. GNase I had a K_m of 0.39 mg ml^{-1} for lentinan, whereas GNase II had a K_m value of 0.92 mg ml^{-1}. On the basis of a molecular mass for authentic lentinan of 381 kDa, as determined by GPC analysis, these finding correspond to substrate concentrations of 1.02 and 2.41 μM.

In order to clarify the mode of action of GNase I and II on lentinan, the identification of the enzymatic degradation products was attempted. Elution patterns of the degradation products on Sephacryl S-300 HR are shown in Fig. 3. Two main peaks were observed on the elution patterns of the enzymatic degradation products of lentinan by GNase I and II even for 0.25 and 48 h (Fig. 3). The higher molecular products were eluted at a fraction of about 400 kDa of molecular mass, which corresponds to that of authentic lentinan. The lower molecular products were eluted at a range of less than 3000 Da. To identify the degradation products that were eluted at less than 3000 Da, they were further subjected to HPLC analysis. Furthermore, a single peak on the chromatogram was detected at a retention time corresponding to that of authentic glucose, as determined by HPLC analysis of the degradation products liberated enzymatically from lentinan (data not shown), and the negative-SIMS mass spectrum of the peak was assigned to that of glucose (data not shown). It was shown that lentinan was degraded by β-glucanases and glucose increased in Fig. 3. These results suggest that both purified β-glucanases acted in an exo-hydrolytic manner.

Table 2
Kinetic properties of purified β-1,3-glucanases from *L. edodes*

Substrate	GNase I	GNase II
Lentinan		
K_m (mg ml^{-1})	0.39	0.92
K_m (μM)[a]	1.02	2.41

Parameters were determined at 40 °C in 30 mM sodium acetate buffer, pH 4.2, using 5.3 and 5.4 munits of GNase I and II, respectively.
[a] Based on a molecular mass for lentinan of 381 kDa by analysis of GPC.

Most fungi appear to secrete both exo- and endo-β-1,3-glucanases (Pitson, Seviour, & McDougall, 1993), and these may act synergistically for the efficient degradation of simple and complex β-D-glucans (Copa-Patinõ, Rodriquez, Reyes, & Perez-Leblic, 1990). However, *L. edodes* contained only the exo type of β-1,3-glucanase (EC 3. 2. 1. 58); this has also been seen in the case of *A. persicinum* β-1,3-glucanases (Pitson et al., 1995). In addition, the specific activity of GNase I in the crude enzyme was higher than that of GNase II (data not shown). Thus, it was thought that GNase I played a more important role in hydrolyzing lentinan than GNase II.

3.2. Pattern of the de novo synthesis of β-1,3-glucanase in *L. edodes* during storage

The synthetic pattern of GNase I was examined in the *L. edodes* fruiting body during storage, because the specific activity of GNase I was higher than that of GNase II in crude enzyme. A western blot analysis demonstrated that GNase I could not be synthesized in the *L. edodes* fruiting body immediately after harvesting (Fig. 4). GNase I was not synthesized in the fruiting body during storage at 1 °C for 7 days. And, it was shown that the enzyme was not synthesized for 3 days during storage at 5 °C. On the other hand, it was already synthesized during storage for 1 day at 20 °C. Then, it reached its highest level of synthesis at 3 days and this level was maintained through the fifth day of storage. Thereafter, the level of GNase I decreased gradually (Fig. 4).

Our previous report demonstrated that β-glucanase activity rapidly increased and peaked after 3 days in a storage at 20 °C. After this activity was kept for 2 days, then it decreased gradually during storage. On the other hand, in a storage at 1 °C, β-glucanase activity was not observed during storage of the mushroom. And, it was only slightly enhanced during storage for 5 days at 5 °C, then increased

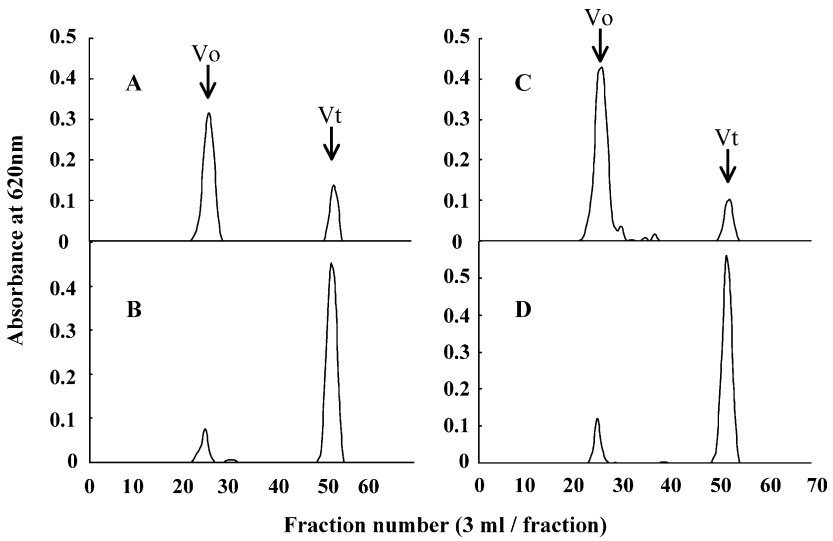

Fig. 3. Gel permeation pattern on a Sephacryl S-300 HR of the enzymatic digests of lentinan by GNase I and GNase II in *L. edodes*. Authentic lentinan was incubated with GNase I for 0.25 h (A), 48 h (B), and with GNase II for 0.25 h (C), 48 h (D). Amount of enzymes used were 5.3 and 5.4 munits for GNase I and II, respectively. Sugar contents were measured by the anthrone–H_2SO_4 method at 620 nm.

after 7 days (Minato et al., 1999a). The pattern of these β-glucanase activities was consistent with that of western blot analysis in *L. edodes* fruiting bodies during storage at 1, 5, and 20 °C in this study. Moreover, it was suggested that the pattern of western blot of GNase I related to β-glucanase activity, because the specific activity of this isoenzyme was higher than that of GNase II in crude enzyme from *L. edodes*. Therefore, these results demonstrate that GNase I can be synthesized de novo in the fruiting body of *L. edodes* under storage conditions at 20 °C, and play an important role in digestion of lentinan during storage of the mushroom.

3.3. The effect of enzymatically fragmented products from authentic lentinan on TNF-α and NO production

When a purified lentinan was incubated with GNase I, the amount of lentinan decreased (Fig. 3A and B). The level of lentinan was initially 500 μg ml^{-1}; the level degraded to 452.8 ± 43.1 μg ml^{-1} after incubation for 0.25 h, 349.4 ± 25.0 μg ml^{-1} after 0.5 h, 178.9 ± 55.9 μg ml^{-1} after 4 h, and finally reached 113.1 ± 14.5 μg ml^{-1} after incubation for 48 h by using ELISA. To investigate the immunomodulating activities of the GNase I-fragmented lentinan products, the amount of TNF-α and NO produced from murine macrophages was measured. TNF-α production decreased with a decrease in lentinan content incubated with GNase I (Fig. 5). TNF-α was released 248.9 ± 83.3 pg ml^{-1} from macrophages stimulated with the mixture without incubation, which contained 500 μg ml^{-1} of lentinan. However, the amount of TNF-α produced from murine macrophages stimulated with the mixture after incubation for 0.25, 0.5, and 4 h decreased to 214.6 ± 67.0, 148.4 ± 59.0, and 68.7 ± 20.8 pg ml^{-1}, respectively. After a 48 h-incubation, TNF-α production decreased to equal level compared with control. NO production also decreased with lentinan degradation by GNase I (Fig. 6). NO was released in an amount of 11.4 ± 2.8 μM from murine macrophages stimulated with 500 μg ml^{-1} of lentinan. NO production decreased with

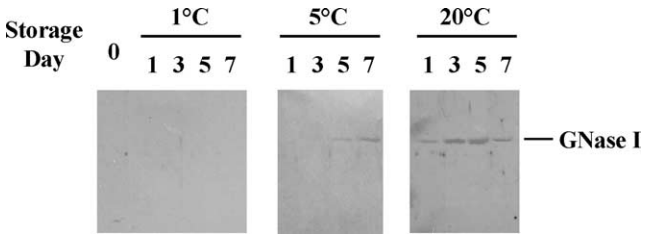

Fig. 4. The pattern of β-1,3-glucanase synthesis in *L. edodes* fruiting bodies by western blotting analysis during storage at different temperature. The fruiting bodies were stored at 1, 5, and 20 °C for 7 days.

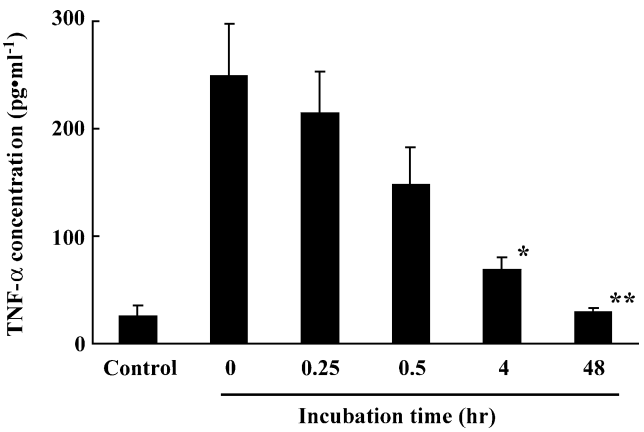

Fig. 5. Effect of enzymatic degradation products of lentinan on TNF-α productions from murine macrophages. Lentinan (500 μg ml^{-1}) was incubated with GNase I (100 units) in 30 mM sodium acetate buffer, pH 4.2 at 40 °C for 0.25, 0.5, 4, and 48 h. All data are means + SD ($n = 3$). Student's *t*-test: $^{*}P < 0.025$ and $^{**}P < 0.02$ against 0 h.

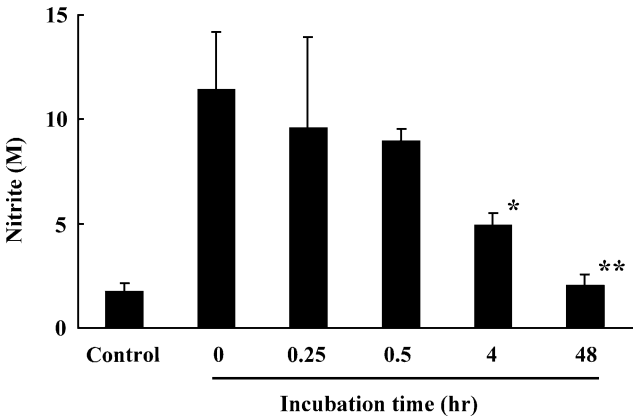

Fig. 6. Effect of enzymatic degradation products of lentinan on NO productions from murine macrophages. Lentinan (500 μg ml^{-1}) was incubated with GNase I (100 units) in 30 mM sodium acetate buffer, pH 4.2 at 40 °C for 0.25, 0.5, 4, and 48 h. All data are means + SD ($n = 3$). Student's t-test: $^{*}P < 0.02$ and $^{**}P < 0.005$ against 0 h.

the elapse of incubation time of authentic lentinan with GNase I, and the amounts of NO were 9.6 ± 2.8, 9.0 ± 2.8, and 5.0 ± 2.8 μM after incubation for 0.25, 0.5, and 4 h, respectively. After a 48 hr-incubation of lentinan with GNase I, the NO level also decreased to equal level compared with control.

It is known that lentinan stimulates macrophages to augment their antitumor functions. The function of macrophages may be comprised of two mechanisms, as follows: one is cell-to-cell contact between macrophages and tumor cells, and another is the release of antitumor factors such as cytokines (TNF-α and interleukin etc.), eicosanoids, reactive oxygen metabolises, and nitrogen intermediates. TNF-α has been recognized as a factor that induces the necrosis of a transplanted murine tumor (Carswell et al., 1975). NO has also recently been identified as a mediator of the antitumor effect of macrophages. We have already demonstrated that the immunomodulating activity of *L. edodes* decreased during storage at 20 °C, due to a decrease in TNF-α and NO productions from murine peritoneal macrophages; such decreases would be related to the decrease in the amount of lentinan by β-glucanases-degradation (Minato et al., 1999b). And, when the equal amount of authentic purified lentinan to that after 48 h treatment with β-glucanase was used, the immunomodulating activity was hardly shown. In the present study, it was demonstrated with certainly that an authentic lentinan was degraded by the purified the β-1,3-glucanase from *L. edodes*, and that the immunomodulating activity of the enzymatical degraded lentinan products diminished (Figs. 5 and 6). These results suggested that the β-1,3-glucanase could degrade lentinan in *L. edodes*, and depleted the immunomodulating activity of *L. edodes* in cytokines productions from murine macrophages during storage.

4. Conclusion

Our previous study showed that the β-glucanase activity increased during storage of *L. edodes* at 20 °C, and the lentinan content and an immunomodulating activity of the mushroom decreased. In this study, it demonstrated that an exo type β-1,3-glucanase was synthesized de novo in *L. edodes* fruiting body during storage at 20 °C. And, this enzyme was associated with diminishment of immunomodulating activities of lentinan in cytokine productions. These findings suggest that the lentinan degradation, which caused by an exo type β-1,3-glucanase, resulted in the diminishment of the immunomodulating activity of *L. edodes* during storage at 20 °C.

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