Establishment of Determination of Antitumor Polysaccharides in Mushrooms by Enzyme Linked Immunosorbent Assay and Its Application

湊, 健一郎

(Degree)
博士（学術）

(Date of Degree)
2000-03-31

(Date of Publication)
2008-06-23

(Resource Type)
doctoral thesis

(Report Number)
甲2070

(JaLCDOI)
https://doi.org/10.11501/3173009

(URL)
https://hdl.handle.net/20.500.14094/D1002070

ESTABLISHMENT OF DETERMINATION OF ANTITUMOR POLYSACCHARIDES IN MUSHROOMS BY ENZYME LINKED IMMUNOSORBENT ASSAY AND ITS APPLICATION

食用キノコ中抗腫瘍性多糖の酵素免疫化学的定量法の確立とその応用

KEN-ICHIRO MINATO

湊 健一郎

CONTENTS

I GENERAL INTRODUCTION

The mushrooms have recently become attractive as functional foods, and their extracts are widely sold as nutritional supplements and touted as beneficial for health (Borchers *et al.*, 1999). The mushrooms are also expected to become source materials for development of drugs. Some papers recently have described that some of isolated and identified substances of higher Basidiomycetes mushrooms origin expressed promising antitumor, immunomodulating, anti-hypercholesteroletic, antiviral, antibacterial, and antiparasitic effects (Wasser and Weis, 1999). In particular, the effective mushrooms against cancers of the stomach, esophagus, lungs, etc. are well-known in the U. S. A., Canada, Russia, Korea, as well as Japan and China. There are about two hundreds species of mushrooms that have been found to markedly inhibit the growth of different kinds of tumors (Chihara *et al.*, 1969, Komatsu *et al.*, 1969, Nanba *et al.*, 1987a and Mizuno *et al.*, 1990a, b). It is considered that searching for new antitumor and other medicinal substances from mushrooms and studying the medicinal value of these mushrooms have become a matter of great significance.

Ikekawa *et al* (1968, 1969) reported that the hot water extracts from some kinds of mushrooms showed significant antitumor activities against implanted tumor of Sarcoma 180 through host-mediated. Many investigators have reported that antitumor substances were isolated and identified from some kinds of the mushrooms, and an antitumor activity of mushrooms was appeared to be attributable to the polysaccharides and

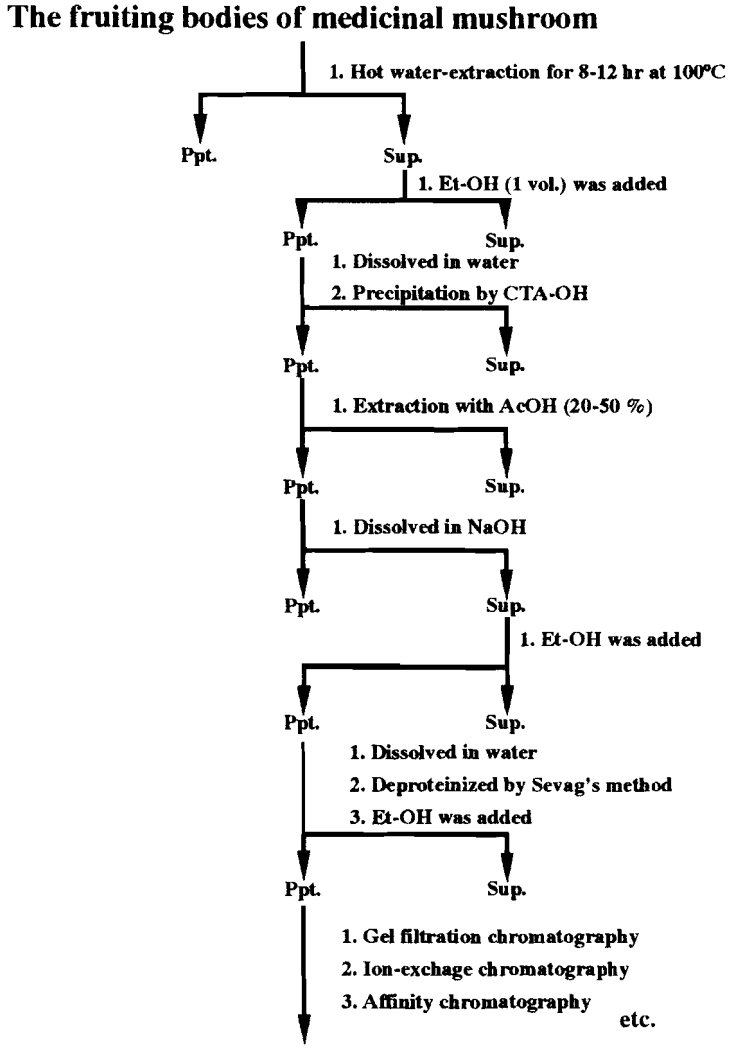
polysaccharide-protein complexes contained in themselves. These antitumor polysaccharides and polysaccharide-protein complexes had been isolated and characterized from many kinds of mushrooms as follows; *Lentinus edodes*, "Shiitake" (Chihara *et al.*, 1969, 1970), *Grifola frondosa*, "Maitake" (Nanba *et al.*, 1987a), *Agaricus blazei*, "Himematsutake" (Mizuno *et al.*, 1990a, b), *Schizophyllum commune*, "Suehirotake" (Komatsu *et al.*, 1969), *Coriolus versicolor*, "Kawaratake" (Tsukagoshi *et al.*, 1984), *Flammulina velutipes* "Enokitake" (Ikekawa *et al.*, 1982), *Auricularia auricula-judae*, "Kikurage" (Misaki *et al.*, 1981), *Ganoderma lucidum*, "Man-nentake" (Mizuno *et al.*, 1984), *Amanita muscaria*, "Benitengutake" (Kiho *et al.*, 1992), *Polyporous confluens*, "Ningyotake" (Mizuno *et al.*, 1992), *Tricholma giganteum*, "Niohshimeji" (Mizuno *et al.*, 1995), *Ganoderma tsugae*, "Songshan Lingzhi" (Wang *et al.*, 1993), *Pleurotus sajor-caju*, "Houbitake" (Zhuang *et al.*, 1993), *Cryptoporus volvatus*, "Hitokuchitake" (Kitamura *et al.*, 1994) and *Sarcodn aspratus*, "Koutake" (Maruyama *et al.*, 1989).

It is well known that the antitumor polysaccharides in these mushrooms enhance the immunomodulating effects in host (Di Luzio, 1985). Hence, these polysaccharides are recognized as a biological response modifier (BRM). Chihara *et al* (1969, 1970) isolated and purified lentinan from the hot water extract of *L. edodes*. Lentinan had a strong antitumor effect; it markedly inhibited the growth of Sarcoma 180 implanted subcutaneously in mice, inducing almost complete regression of tumors at doses of 1 $mg \bullet kg^{-1} \times 10$ without toxicity. It has been also reported that lentinan showed prominent antitumor activity in murine allogeneic and syngeneic host (Zákány *et al.*, 1980, Rose *et al.*, 1984 and Suga *et al.*,

1984). It is widely accepted that the activated immunocompetent cells, such as macrophages, cytotoxic T cells, and natural killer (NK) cells, in host usually play important roles in tumor immunity (Maeda *et al.*, 1974 and Hamuro *et al.*, 1978). These reports suggest that the lentinan acts as an immunomodulator to develop tumor immunity against allogeneic and some syngeneic tumors. In addition, Nanba *et al* (1987b, c) reported that the extract containing lentinan from *L. edodes* fruiting bodies showed antitumor effects on allogeneic and syngeneic tumors after oral administration.

Grifolan has been isolated and purified from *G. frondosa* mycelium (Ohno *et al.*, 1985). And, the antitumor β-glucan (designated as GGF) was extracted from *G. frondosa* fruiting body by Nanba *et al* (1987a). The grifolan administered *in vivo* enhanced immunoreactivity, inducing activation of macrophages, cytotoxic T cells and NK cells (Ohno *et al.*, 1986a, b, Takeyama *et al.*, 1987, Adachi *et al.*, 1989 and 1994). It has been also reported that GGF have strong host-mediated antitumor activity against various tumors (Nanba *et al.*, 1987a). It has been also reported that antitumor poysaccharides such as schizophyllan from *S. commune*, PSK from *C. versicolor*, SSG from *Sclerotinia sclerotiorum* (Ohno *et al.*, 1986), Flo-a-β fraction from *A. blazei* (Mizuno *et al.*, 1990a) and H-3-B fraction from *C. volvatus* (Kitamura *et al.*, 1994) were isolated and purified. Indeed, lentinan and schizophyllan have been applied clinically (Azuma, 1987).

Many investigators have reported that these antitumor polysaccharides played an important role in immune system. And, the antitumor and immunomodulating activities of these polysaccharides have been extensively studied. However, little has been reported



Scheme I. Extraction of an antitumor polysaccharide by traditional method as described by Chihara *et al* (1970) and Nanba *et al* (1987a)

whether the contents and antitumor activities of these polysaccharides changed during growth and storage of the mushrooms. Although the analytical methods of polysaccharides have improved considerably, it is still difficult to purify and quantify the polysaccharides as summarized in Scheme I (Chihara *et al.*, 1970 and Nanba *et al.*, 1987a). The polysaccharides are extracted from the fruiting bodies with hot water for long time (8 to 15 hr), and then the

polysaccharide fractions were obtained by precipitation with ethanol and the detergent. Furthermore, the fractions were extracted under acidic conditions. And then, the extracts were separated and purified by ion-exchange and gel chromatography, and finally the purified powders of antitumor polysaccharides were obtained. Thus, it is very tedious to obtain the polysaccharides reproducibly, and difficult to determine them exactly. This seems a reason why no paper has reported how the contents of antitumor polysaccharides changed in the mushrooms during growth and storage of the fruiting bodies. However, immunoassay seems to be a simple and exact method that may make possible a more precise analysis of a polysaccharide (Takano *et al.*, 1988, Douwes *et al.*, 1996). Indeed, some papers have reported that antibodies against several polysaccharides were prepared and applied for detection and determination of the polysaccharides (Meikle *et al.*, 1991, Kikuchi *et al.*, 1993). Recently, Tabata *et al* (1990) reported that they had been able to prepare polyclonal antibodies against an antitumor polysaccharide, schizophyllan from *S. commune*. And, Hirata *et al* (1993) reported that they had succeeded in preparing a monoclonal antibody against schizophyllan. Adachi *et al* (1994) had reported about the preparation of polyclonal antibodies to grifolan-BSA (bovine serum albumin) conjugate. In order to establish the exact and simple analysis for the antitumor polysaccharides in mushrooms by an immunochemical assay, the author planned to prepare a few anti-polysaccharide antibodies, and then attempted to apply the enzyme linked immunosorbent assay (ELISA) to determine the polysaccharides in the mushrooms using these antibodies, in this study. Moreover, in order to elucidate whether the contents of antitumor polysaccharides change during storage of

the mushrooms, the changes in the contents of a few antitumor polysaccharides were investigated under a few different storage conditions of mushrooms.

In Chapter II, antibodies against lentinan and GGF, as an antitumor polysaccharide, were prepared. The specificities and characterizations of these antibodies were examined to establish the inhibition assay of ELISA as an effective method for quantification of antitumor polysaccharides in the mushrooms. In Chapter III, the ELISA inhibition assay was applied to determine the lentinan-like and GGF-like polysaccharides in some edible mushrooms. And, it was investigated the process of accumulation of lentinan and GGF during growth of *L. edodes* and *G. frondosa*, respectively. Moreover, the localization of lentinan in *L. edodes* tissue was examined by immunohistochemical analysis using anti-lentinan antibodies. In Chapter IV, the changes in the contents of lentinan and GGF during storage accompanied by the quality reduction of the fruiting bodies were examined by the inhibition assay of ELISA. Simultaneously, the change in the productions of tumor necrosis factor-α (TNF-α) and nitric oxide (NO) from stimulated murine macrophages as the indicator of the immunomodulating activity of *L. edodes* with decrease in lentinan contents during storage of fruiting bodies was examined. Since antitumor polysaccharides do not show any direct cytotoxicity against tumor cells (Maeda and Chihara, 1973), they may exert their effects through activation of various effector cell types in the immune system (Wong *et al.*, 1994a, b and c), and their effects are recognized as immunomodulating or immunostimulating activities. Recently, it has been reported that these immunomodulating polysaccharides stimulated immunocomplement cells such as macrophages, and could induce

the productions of some kinds of cytokines that attacked targeting tumor cells (Liu *et al.*, 1996, Okazaki *et al.*, 1995 and Sakurai *et al.*, 1994). It has been reported that murine peritoneal macrophages stimulated with lentinan produced several cytokines, TNF-α and NO (Kerékgyártó *et al.*, 1996, Irinoda *et al.*, 1992). TNF-α is recognized as the primary cytokine produced mainly by activated macrophages; it is an important host defense molecule that affects tumor cells (Carswell *et al.*, 1975). The works in several disciplines have converged to establish NO as a major messenger molecule regulating immune function in host (Lowenstein and Snyder, 1992).

In Chapter IV, the author also investigated the relationship between the decrease of lentinan content and the increase of β-glucanase activity. As it was seemed that β-1, 3-glucanase activity was associated with lentinan degradation during storage of *L. edodes*, these enzymes were isolated and purified, and examined some properties of them in Chapter V. And, in order to clarify the degradation mechanism of lentinan, the change pattern of the β-1, 3-glucanase synthesis in fruiting body of *L. edodes* during storage was investigated by western blotting analysis. In Chapter VI, the author discussed the utility of ELISA as the strategy for quantification of antitumor polysaccharides in mushrooms, and the decrease in lentinan examined by this ELISA method during storage of *L. edodes* and its degradation mechanism, on the basis of all data obtained in this study.

II ESTABLISHMENT OF ELISA SYSTEM FOR DETERMINATION OF THE ANTITUMOR POLYSACCHARIDES, LENTINAN AND GGF

II – 1 INTRODUCTION

It is known that lentinan is a β-1, 6 branched β-1, 3-glucan isolated from *Lentinus edodes* "Shiitake" fruiting body (Chihara *et al.*, 1969 and 1970), and GGF is a β-1, 3 branched β-1, 6-glucan from *Grifola frondosa* "Maitake" fruiting body (Nanba *et al.*, 1987a). They show prominent antitumor activities in murine allogeneic and syngeneic hosts, and their immunopharmacological properties are well characterized as T-cell-oriented immunopotentiator in which macrophage functions take some part (Maeda *et al.*, 1971, Maeda and Chihara, 1971 and 1973).

It can be assumed that the establishment of exact method for the determination of an antitumor polysaccharide leads to the clarification of the behavior of the polysaccharide in the mushroom. In this chapter, thus, the author prepared the polyclonal antibodies against lentinan and GGF, and planned to make use of enzyme-linked immunosorbent assay (ELISA) to quantify simply and exactly an antitumor polysaccharide in the mushroom fruiting bodies.

II – 2 MATERIALS AND METHODS

Antitumor polysaccharides

Purified lentinan was kindly gifted by Ajinomoto Co. (Tokyo, Japan). GGF was isolated from the fruiting body of *G. frondosa* according to the method of Nanba *et al*

(1987a). The lyophilized powder of *G. frondosa* (50 g) was mixed with 1 liter of distilled water. The mixture was heated at 100°C for 10 hr and filtrated, then the supernatant and equal volume of EtOH were combined and left at 4°C for 12 hr. The precipitate was separated by centrifugation (10,000 × g, 30 min) and dissolved in distilled water. 25 % cetyltrimethylammonium hydroxide was added dropwise to the aqueous solution with stirring until no more precipitates was formed (pH exceed 12). All the precipitates were collected by centrifugation (10,000 × g, 30 min), and suspended in 1.2 liters of 20 % acetic acid, and stirred for 5 min at 0°C. And then, the precipitates were obtained by centrifugation (10,000 × g, 30 min). Furthermore, the precipitate was washed with 1 liter of 50 % acetic acid for 3 min at 0°C by stirring in Waring blender, centrifuged (10,000 × g, 30 min), and fractionated into insoluble and soluble parts. The insoluble part was dissolved in 2 liters of 6 % NaOH, contaminants were removed by centrifugation (10,000 × g, 30 min). 4 volumes of EtOH were added to the supernatant, and the precipitate formed was collected, washed twice with EtOH and then once with ether, and dried under vacuum to give powdered fraction. The fraction obtained was dissolved in water and the solution was treated with a 2:1 (v/v) mixture of $CHCl_3$ and MeOH to remove protein. The aqueous fraction was recovered, and 4 volumes of EtOH were added to the solution to obtain the precipitate. The precipitate was further purified by anion exchange (DEAE-Sepharose CL-6B, ϕ 2.6 × 34 cm, Amersham Pharmacia Biotech, Amersham, UK) and gel filtration (Sepharose CL-4B, ϕ 2.5 × 45 cm, Amersham Pharmacia Biotech) chromatography. The light brown powder was obtained as GGF.

Animal

New Zealand White rabbit (female, 16 weeks of ages) was purchased from Japan SLC Co. (Shizuoka, Japan).

Other polysaccharides

Laminarin (β-1, 3-glucan) and Dextran (α-1, 6-glucan, Mw; 1.1×10^4) were purchased from Sigma Chemical (St. Louis, MO). Pullulan (α-1, 6-glucan, Mw; 85.3×10^4) was purchased from Showa Denko Co. (Tokyo, Japan). Galactan extracted from Gum Arabic was purchased from Aldrich Co. (Milwaukee, WI). Amylose (α-1, 4-glucan, Mw; 2.9×10^4) was purchased from Nacalai Tesque Co. (Kyoto, Japan). Pustulan (β-1, 6-glucan, Mw; 2.0×10^4) was purchased from Calbiochem Co. (La Jolla, CA).

Preparation of anti-lentinan and anti-GGF antibodies

Lentinan and GGF (0.5 mg) were dissolved in 1 ml of phosphate buffered saline (PBS, pH 7.2). These solutions were emulsified with an equal volume of Freund's complete adjuvant, respectively. The emulsions were injected subcutaneously at 10 different sites in the back of the rabbits, individually. Half volume of lentinan or GGF in first dosage was boosted again two weeks after first injection. After the boost, the blood was collected to obtain anti-lentinan and anti-GGF sera several times every one week. The sera were stored at -80°C until use. After measurement of these antisera titers, the rabbits were exsanguinated. Finally, IgG fractions in the obtained antisera were separated by

Protein G (Amersham Pharmacia Biotech) affinity chromatography, and these fractions were used as anti-lentinan and anti-GGF antibodies, respectively.

Titer of the antisera

The titers of the anti-lentinan and anti-GGF antisera, respectively, were measured by the direct assay of ELISA as following. The solution (100 μl) of antigens (lentinan or GGF in PBS, 50 $\mu g \cdot ml^{-1}$) were placed in micro-titer wells (96 wells, Sumitomo Bakelite Co., Tokyo, Japan), and coated overnight at 4°C. Then, all wells were filled with PBS containing 1 % skim milk for blocking and kept for 2 hr at 20°C. After the blocking solution was removed by decantation and washed three times with PBS-Tween (0.02 % Tween 20 in PBS), 100 μl of from 1:1,000 to 1:1,000,000 diluted antisera was added to the micro-titer wells. The wells were incubated for 1.5 hr at 20°C. After washing another three times with PBS-Tween solution, 100 μl of 1:2,000 diluted horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (Wako Pure Chemical Industries LTD., Osaka, Japan) was added to the wells and incubated for 1.5 hr at 20°C. After frequent washing and coloration with H_2O_2-*O*-phenylenediamine (100 μl), the absorbance at 492 nm was measured by a microplate reader (MTP-120, Corona Electric Co. LTD., Japan).

ELISA inhibition assay

An inhibition assay of ELISA was used to confirm the higher specificity of the anti-lentinan and anti-GGF antibodies, respectively, against several polysaccharides. The

solution of antigens (lentinan or GGF in PBS, 50μg•ml^{-1}) was coated on micro-titer wells overnight at 4°C, and thereafter the wells were blocked with 1 % skim milk solution in PBS-Tween. In parallel, the each antibody was incubated with the samples or various standard polysaccharides dissolved in PBS for 30 min at 4°C. After washing the wells with PBS-Tween, the solution (100 μl) mixed with antibodies and samples were added to the wells. The plates were incubated for 1 hr at 20°C and washed with PBS-Tween. An HRP-conjugated goat anti-rabbit IgG antibody was added, and the plates were incubated for 1.5 hr at 20°C. After washing and coloration with H_2O_2-*O*-phenylendiamine (100 μl), the absorbance at 492 nm was measured.

Quantification of lentinan contents in L. edodes extract by ELISA

Crude antitumor polysaccharides fractions from *L. edodes* was prepared essentially according to Chihara *et al* (1970). The fresh fruiting bodies (20 g) were homogenated with liquid nitrogen by a Warning blender and lyophilized. The lyophilized samples (2 g) were extracted with hot water (100 ml) for 8 hr. The suspensions were filtrated to remove insoluble matters. The crude lentinan fractions were obtained by precipitation with equal volume of EtOH to the filtrate. The content of lentinan in extract was measured by the direct assay and the inhibition assay of ELISA.

II – 3 RESULTS

Titer of anti-lentinan antiserum

Anti-lentinan antiserum was raised in a rabbit by immunization. The titers of anti-lentinan antiserum as evaluated by a direct assay of ELISA are shown in Table II-1. The serum before the infection did not response against lentinan. The titer of the antisera were expressed the dilution magnification that was given an equal absorbance of non-immunization serum (Table II-1). The titer of anti-lentinan antisera increased to the maximum in two weeks after first infection. After the booster dose, it kept the maximum value for about one month (Figure II-1). Therefore, the anti-lentinan antiserum at day 35 was used for further experiments.

Table II-1. Reactivity of the rabbit anti-lentinan serum as assessed by ELISA direct assay.

Dilution of antisera	Absorbance at 492nm
1:100	1.189
1:1,000	0.163
1:10,000	0.020

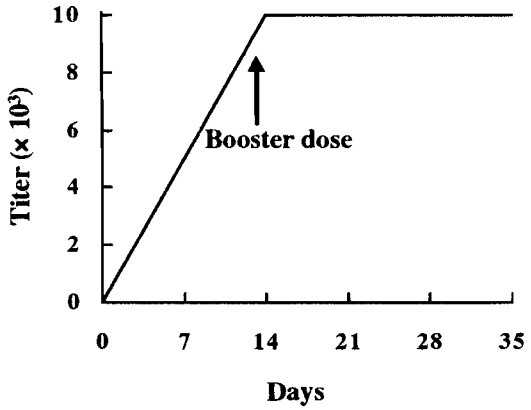


Figure II-1. The titer of anti-lentinan antisera. The antisera were collected at 14, 21, 28 and 35 days.

The determination of lentinan by ELISA

As shown in Figure II-2, a standard curve was obtained by the direct assay of ELISA. The linear calibration curve was obtained in the range of 0.5 to 2 $\mu g \cdot ml^{-1}$. Table II-2 shows lentinan content in *L. edodes* extract as assessed by ELISA direct assay. Although

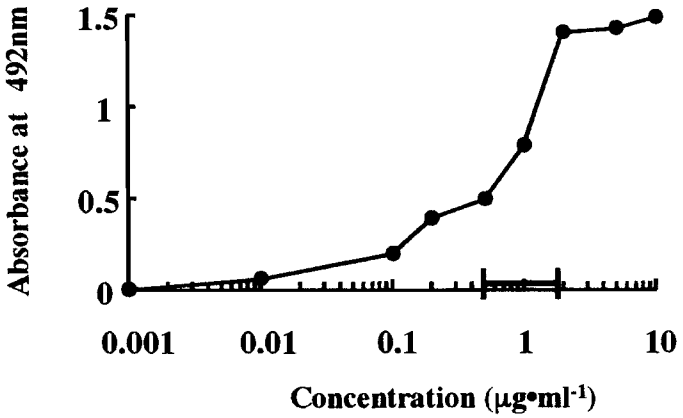


Figure II-2. Calibration curve for lentinan by direct assay of ELISA using anti-lentinan antibody. The bar showed the range of linear calibration curve.

the concentration of *L. edodes* extract was altered, lentinan content did not change (approximately 2 $\mu g \cdot ml^{-1}$ extract). This result suggested that lentinan in the mushroom extract could not be determined by this method. Then, the author planned to determine lentinan by the inhibition assay of ELISA as another application.

Table II-2. Lentinan content in *L. edodes* extract as assessed by ELISA direct assay.

Dilution of extract	Lentinan content ($\mu g \cdot ml^{-1}$ extract)
1:100	2.1 ± 0.5
1:500	2.3 ± 0.5
1:1,000	2.3 ± 0.4

As shown in Figure II-3, a standard curve was calculated for the determination of lentinan by the inhibition assay of ELISA. The anti-lentinan antibodies reacted with lentinan in the range of 1 to 500 $\mu g \cdot ml^{-1}$. In the range of 2 to 50 $\mu g \cdot ml^{-1}$ of lentinan, the linear calibration curve was obtained by the inhibition assay of ELISA. The content of lentinan in the *L. edodes* extracts could be measured with dose-dependency by this method (Table II-3). This result suggested that lentinan contents of the mushroom extract could be determined by the inhibition assay of ELISA.

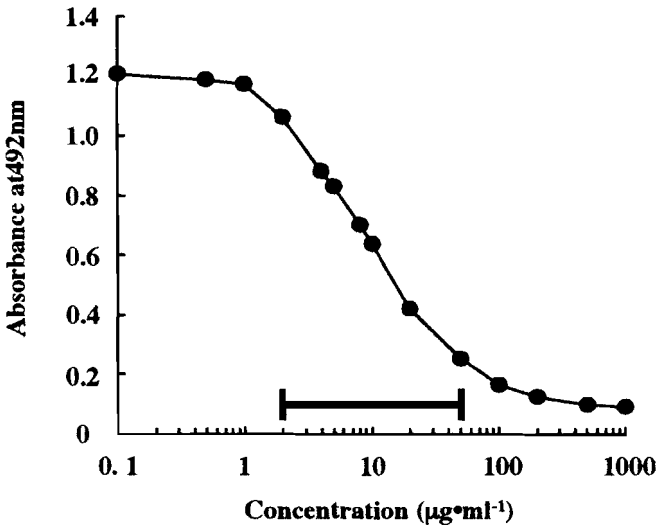


Figure II-3. Inhibition-dilution curve for lentinan by ELISA using anti-lentinan antibody. The bar showed the range of a linear calibration curve.

Table II-3. Lentinan content in *L. edodes* extract as assessed by ELISA inhibition assay.

Dilution of extract	Lentinan content ($\mu g \cdot ml^{-1}$ extract)
1:100	29.2 ± 4.6
1:500	12.1 ± 0.7
1:1,000	2.9 ± 0.6

Specificity of anti-lentinan antibody

The specificity of the anti-lentinan antibody as assessed by ELISA inhibition assay was examined by using several authentic polysaccharides as shown in Figure II-4. The

anti-lentinan antibody showed significant reaction with only lentinan, and did not react with laminarin, β-1, 3-glucan straight chain that composed of the main chain in lentinan, and galactan. However, it did not recognize α-glucose-bonds such as dextran (α-1, 6-glucan) and amylose (α-1, 4-glucan). This result suggested that the inhibition assay of ELISA were sufficiently applied to measure the lentinan contents in the mushrooms.

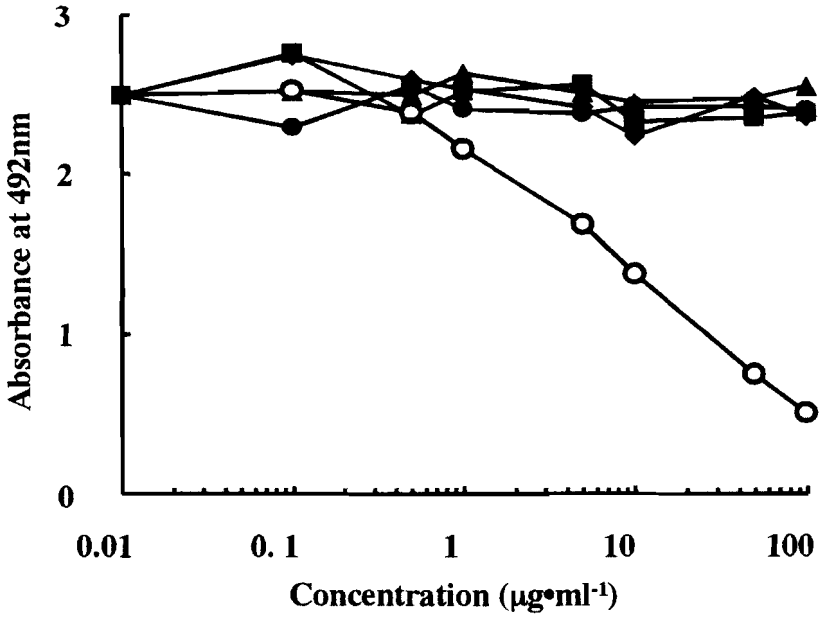


Figure II-4. Specificity of anti-lentinan antibody to polysaccharides. The specificity of antibody against (O); Lentinan, β-1,3-glucan, (■); Amylose, α-1,4-glucan, (◆); Galactan, (●) Laminarin, β-1,3-glucan (▲); Dextran, α-1,6-glucan.

Titer of anti-GGF antiserum

Anti-GGF antiserum was raised in a rabbit by immunization. The absorbance at 492 nm for the antiserum as evaluated by ELISA is shown in Table II-4. The serum before the infection did not respond to GGF. The titer of anti-GGF antiserum was increased to

maximum within two weeks after first infection, and it was kept high level to day 28 (Figure II-5). Therefore, the antiserum at day 28 was used for further experiment.

Table II-4. Reactivity of the rabbit anti-GGF serum as assessed by ELISA direct assay.

Dilution of antisera	Absorbance at 492nm
1:1,000	1.229
1:10,000	0.275
1:100,000	0.039

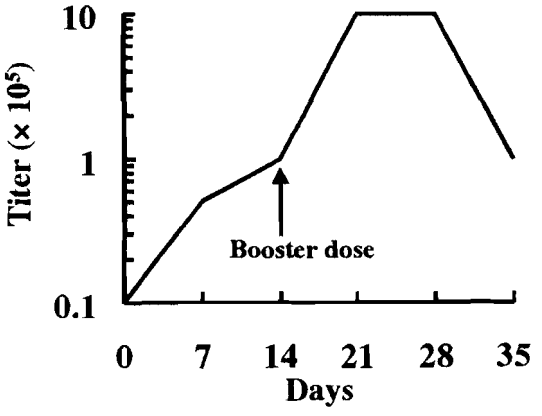


Figure II-5. The titer of anti-GGF antisera. The antisera were collected at 14, 21, 28 and 35 days.

As the inhibition assay of ELISA was available to the determination of lentinan as described above, standard curve was calculated for the determination of GGF by the same

method. As shown in Figure II-6, the anti-GGF antibody reacted with GGF in the range of 0.5 to 100 $\mu g \cdot ml^{-1}$. A linear calibration curve was obtained in the range of 0.5 to 10 $\mu g \cdot ml^{-1}$ of GGF. This result showed that GGF contents might be measured by using the anti-GGF antibody prepared in this experiment

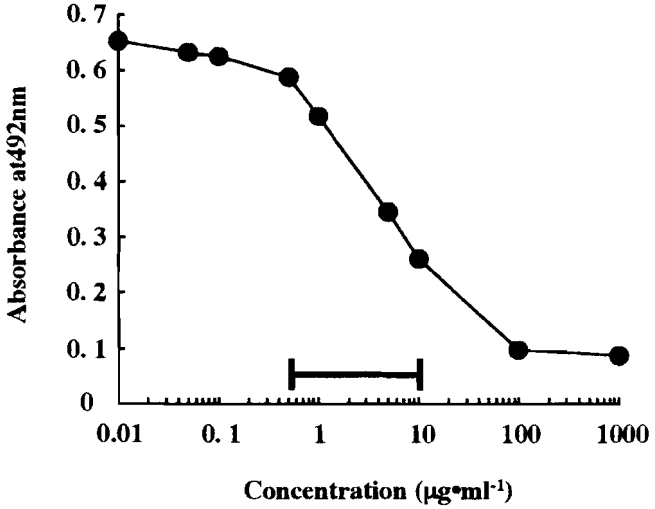


Figure II-6. Inhibition-dilution curve for GGF by ELISA using anti-GGF antibody. The bar showed the range of a linear calibration curve.

Specificity of anti-GGF antibody

The specificity of the anti-GGF antibody to some polysaccharides using the inhibition assay of ELISA is shown in Figure II-7. An anti-GGF antibody reacted with GGF significantly, and slightly with lentinan. However, the anti-GGF antibody did not recognize laminarin and pustulan (a straight chain of β-1, 6-glucan), and pullulan (a straight chain of α-1, 6-glucan). It was proved that GGF content in the mushroom could be sufficiently measured by ELISA inhibition assay.

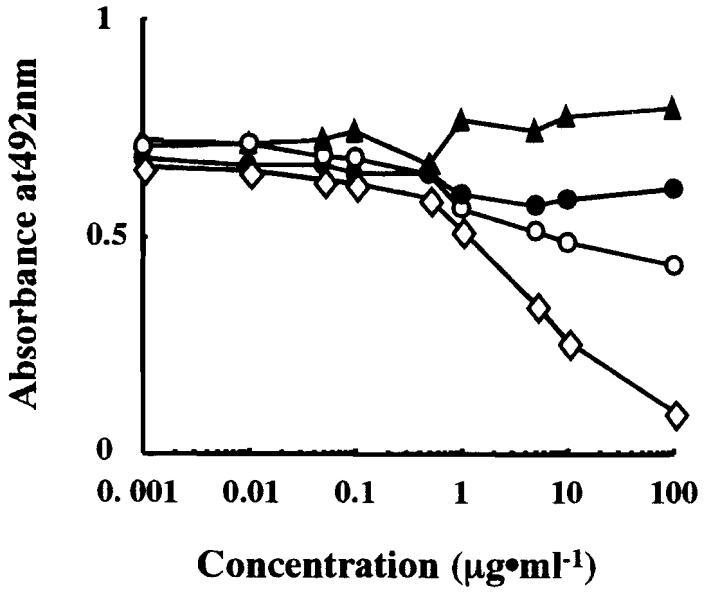
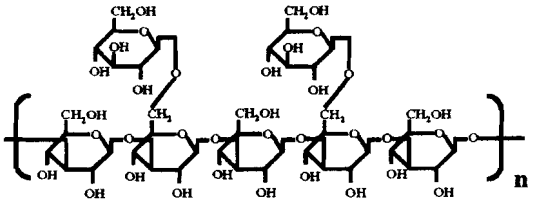


Figure II-7. Specificity of anti-GGF antibody to polysaccharides. The specificity of antibody against (◇); GGF, β-(1, 6)-glucan, (○); Lentinan, β-(1, 3)-glucan, (●); Laminarin, β-(1, 3)-glucan, (▲); Pullulan, α-(1, 6)-glucan.

II – 4 DISCUSSION

As shown in Figure II-8, lentinan has two branches for every five D-glucopyranosyl residues at *O*-6 (Chihara *et al.*, 1970, Sasaki and Takasuka, 1976, Saito *et al.*, 1977, 1979). And, GGF has β-1, 3-glucose side chains on the β-1, 6-glucose main chains (Nanba *et al.*, 1987). The average molecular weights of lentinan and GGF are deduced as 400,000 and 2,000,000, respectively. The average molecular weight of lentinan and purified GGF was determined to be ca. 380,000 and 2,000,000, respectively, by GPC analysis and gel filtration on Sepharose CL-4B using following Blue Dextran (Mw; 2,000,000) and standard pullulan: P-800 (Mw; 850,000), P-400 (Mw; 380,000) and P-200 (Mw; 186,000). These average molecular weights coincided with the previous data (Nanba *et al.*, 1987a).

Lentinan



GGF

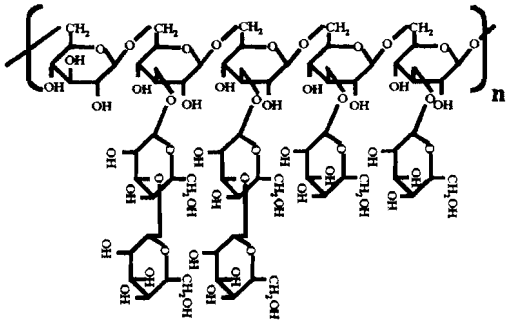


Figure II-8. The structures of antitumor polysaccharides.

The antitumor mechanism of these glucans is thought to be by activation of immunoresponses in the host (Dennert and Tucker, 1973, Dresser and Phillips, 1974, Haba *et al.*, 1976, Hamuro *et al.*, 1978, Zákány *et al.*, 1980a, b and 1983, Hamuro and Chihara, 1984, Ohno *et al.*, 1985 and 1986a, b, Takeyama *et al.*, 1987 and Adachi *et al.*, 1989 and 1994). However, it was insufficiently clarified whether the contents of these antitumor glucans changed in fruiting bodies during storage of the mushrooms. In order to elucidate this problem, it is necessary to establish and improve an exact and easy determination method for an antitumor polysaccharide in mushroom fruiting bodies. Therefore, in this chapter, the author attempted to prepare the antibodies against targeting polysaccharides (lentinan and GGF) and establish ELISA for determination of an antitumor polysaccharide.

Anti-lentinan and anti-GGF antibodies were raised in rabbits by immunizations

(Figures II-1 and 5 and Tables II-1 and 4). The author attempted to use direct assay of ELISA for determination of the antitumor polysaccharides as the easiest method. Although liner calibration curve of lentinan was obtained in range of 0.8 to 2.0 $\mu g \cdot ml^{-1}$ (Figure II-2), it was failed to measure lentinan contents in *L. edodes* extract specifically (Table II-2). This reason was considered that the target polysaccharides in the extracts did not bind on the ELISA plate with dose dependency. These results demonstrated that ELISA direct assay was not applicable to determine the antitumor polysaccharides in mushroom extracts. The author attempted to determine the polysaccharide by ELISA inhibition assay, though it seemed to be a little bit complicated.

The reliable and reproducible standard curves for determination of lentinan and GGF contents were obtained by the inhibition assay of ELISA using these two antibodies (Figures II-3 and 6). And the anti-lentinan and anti-GGF antibodies showed specific reactions with lentinan and GGF, respectively (Figures II-4 and 7). The specificity of the anti-lentinan antibodies was shown in Figure II-4. The antibodies showed the reactivity against only lentinan but not against other polysaccharides at all. Especially, it did not react with laminarin, which composed of a straight chain of β-1, 3 glucan, the backbone of lentinan. This seemed to indicate that the anti-lentinan antibodies might just recognized mainly β-1, 6-linked glucose branches repeated at regular proportion in β-1, 3-glucopyranoside main chains. In addition, lentinan content in extract from *L. edodes* could be measured with dose dependency by the inhibition assay of ELISA (Table II-3). On the other hand, anti-GGF antibodies recognized GGF significantly (Figure II-7). It did not recognized

laminarin and pustulan, indicating that this antibody may recognize mainly a certain core of the ratio of β-1, 6-linked glucose to β-1, 3-linked glucose residues.

The anti-GGF antibody also recognized slightly with high concentration of lentinan (100 $\mu g \cdot ml^{-1}$). As the structure of lentinan is similar to GGF (Figure II-8), the anti-GGF antibodies might react with lentinan at high concentrations. The average molecular weight of GGF and lentinan are 2,000,000 and 400,000, respectively, meaning that the specificity of this antibody against GGF is approximately a 5-fold molar ratio compared to lentinan when ELISA inhibition assay of GGF and lentinan shows the same values (Figure II-7). These results proved that lentinan and GGF contents could be sufficiently measured by the inhibition assay of ELISA using the anti-lentinan and anti-GGF antibodies, respectively, prepared in this experiment.

Another method used to measure β-1, 3-glucans with a glucan-reactive preparation of *Liminus* amebocyte lysate (LAL) has recently been described by Rylander *et al* (1992), and Obayashi *et al* (1995). The reactivities of several β-1, 3-glucans in this assay were reported earlier (Mikami *et al.*, 1982, Roslansky and Novitsky, 1991, Tanaka *et al.*, 1991 and Aketagawa *et al.*, 1993, Nagi *et al.*, 1994). Although the glucan-reactive LAL test is very sensitive (1 to 10 pg of β-1, 3 glucan per ml), it probably is not highly specific since it also reacts with other glucans such as glycophoran (β-1, 6 glucan), and with D-mannan and dextran (Mikami *et al.*, 1982 and Tanaka *et al.*, 1991).

In conclusion, the inhibition assay of ELISA was the best suitable method for the determination of antitumor polysaccharides in the mushrooms specifically and

reproducibility in some determination analysis for polysaccharides. Hence, the author used the inhibition assay of ELISA for the determination of antitumor polysaccahrides in following the Chapter III, IV and V.

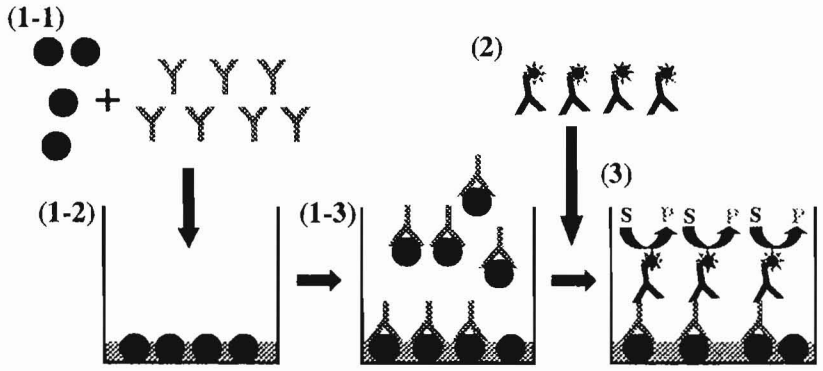


Figure II-9. ELISA inhibition assay

● ; antigen Y ; primary antibody Y ; Secondary antibody

✸ ; HPO (horseradish peroxidase)

(1) Primary antibody incubation.

1-1. Incubate the sample in diluted primary antibody for 30 min at 4°C.

1-2. Incubate the coating well with the mixture of sample and primary antibody for 1 hr at 20°C.

1-3. Rinse the well to remove the conjugations of sample and antibody.

(2) Secondary antibody incubation.

Incubate the well in the diluted HRP labelled secondary antibody for 1.5 hr at 20°C.

(3) Detection.

After coloration with H_2O_2-*O*-phenilenediamine for 10 min at room temperature, absorbance at 492 nm is measured.

III APPLICATION OF IMMUNOCHEMICAL ASSAY

III – 1 INTRODUCTION

Many investigators has reported that several different polysaccharide antitumor agents have been developed from the fruiting body, mycelia and culture medium of various mushrooms (Wasser and Weis, 1999 and Borchers *et al.*, 1999). *Sarcodon aspratus*, "Koutake" (Maruyama *et al.*, 1989 and Mizuno *et al.*, 1999), *Agaricus blazei*, "Himematsutake" (Mizuno *et al.*, 1990 a, b, Ito *et al.*, 1997 and Mizuno *et al.*, 1998), *Agaricus bisporus*, "Tsukuritake" (Nanba and Kuroda, 1988) and *Flammulia velutipes*, "Enokitake" (Leung *et al.*, 1997) contained the polysaccharides that showed significant antitumor activities. In Chapter II, the author succeeded in preparing the two antibodies which reacted specifically with two antitumor polysaccharides, lentinan and GGF, respectively, and the inhibition assay of ELISA was found to be the validest method for the determination of antitumor polysaccharides among the immunoassays. In this chapter, the author attempted to apply the inhibition assay of ELISA to the determination of antitumor polysaccharides in fruiting bodies of some kinds of mushrooms. Moreover, in order to confirm the growth stage when the contents of antitumor polysaccharides are accumulated at highest level in the mushrooms, the changes in the contents of lentinan and GGF were examined during growth of *L. edodes* and *G. frondosa*, respectively. In addition, in order to elucidate the site where an antitumor polysaccharide localized in the fruiting body, the author attempted to develop a novel method that applied the reactivity of anti-lentinan

antibodies with anatomical prints of *L. edodes* tissue on nitrocellulose membrane.

III – 2 MATERIALS AND METHODS

Mushrooms.

Lentinus edodes cultivated in Ohya, Hyogo was used. *Meripilus giganteus*, "Tonbimaitake", *Panellus serotinus*, "Mukitake", *Sarcodon aspratus* and *Lactarius hatsudake*, "Hatsudake" were collected at the seminar forest of Mie University. *Agaricus brasei* was kindly provided by Iwade Mushroom Institute, Mie. *Agaricus bisporus* and *Flammulina velutipes* were purchased from a local market. *Ramaria botrytis*, "Houkitake" was kindly provided by Sakekawa Agricultural Cooperative Association, Yamagata. *Hygrophorus russula*, "Sakurashimeji" was collected in Yamadera, Yamagata. *Grifola frondosa* was kindly provided by Sanda Mushroom Center, Nisshoku Kosan Co., Hyogo, and Yukiguni Maitake Co., Niigata.

Lentinan-like and GGF-like polysaccharides contents in fresh mushrooms

Crude antitumor polysaccharide fractions from various mushrooms were prepared essentially according to Chihara *et al* (1970). The fresh fruiting bodies (20 g) of each mushroom were homogenated with liquid nitrogen by a Waring blender and lyophylized. The lyophilized samples (2 g) were extracted with hot water (100 ml) for 8 hr. The suspensions were filtered to remove insoluble matters. The crude polysaccharide fractions were obtained by precipitation with equal volume of EtOH to the filtrate. The contents of

lentinan-like or GGF-like polysaccharides in fresh mushrooms were measured by the inhibition assay of ELISA as described in Chapter II.

Immunoblotting analysis in mushroom tissue

L. edodes fruiting body was sliced with a razor blade. The nitrocellulose paper (Amersham Pharmacia Biotech) was soaked in 0.2 M $CaCl_2$ for 30 min, and dried on paper towels. Fresh cut tissue was washed in distilled water for 3 s, dried on kimwipes, and blotted on the nitrocellulose membrane for 30 s. The membrane was immediately dried with warm air. And then, the membrane was blocked in 20% skim milk in PBS-Tween overnight at 4°C, treated with the primary anti-lentinan antibodies in PBS-Tween for 1 hr at room temperature. After washed with PBS-Tween three times for 5 min, the membrane was treated with HRP-conjugated goat anti-rabbit IgG antiserum as the secondary antibody for 30 min at room temperature. After washed five times for 5min, binding of secondary antibody was detected with the enhanced chemiluminescence detection method (Amersham Pharmacia Biotech).

III-3 RESULTS

The contents of lentinan in various fresh mushrooms

The contents of lentinan or lentinan-like polysaccharides in mushrooms were calculated by inhibition assay of ELISA. As shown in Table III-1 middle column, the content of lentinan in *L. edodes* was highest and was 3.5 $mg \bullet g^{-1}$ fresh weight (f.w.). In the

other mushrooms tested, the contents of lentinan-like polysaccharide were 2.5, 2.3 and 2.2 mg•g^{-1} f.w. in *F. velutipes*, *L. hatsudake* and *S. aspratus*, respectively. In contrast, those in *M. giganteus*, *P. serotinus*, *H. russula* and *G. frondosa* were less than those in *F. velutipes*, *L. hatsudake* and *S. aspratus*, these being 1.0, 0.9, 0.8 and 0.9 mg•g^{-1} f.w., respectively. However, the lentinan-like polysaccharides were not detected in *A. brazei*, *A. bisporus* and *R. bortryis* at all.

Table III-1. The contents of lentinan and GGF in mushrooms as assessed by ELISA using anti-lentinan and anti-GGF antibodies.

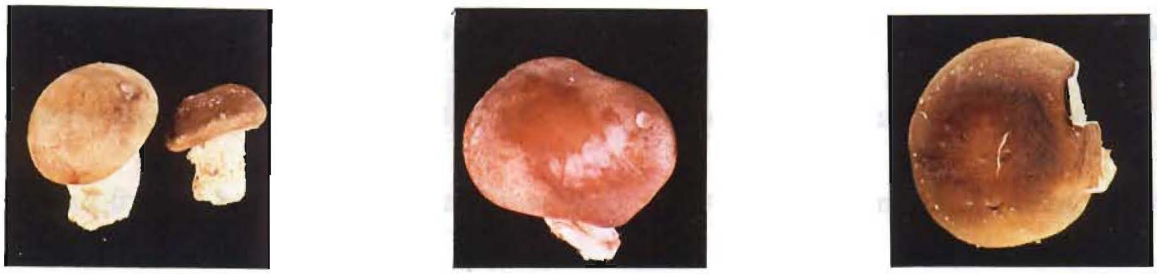
Mushrooms	Content of polysaccharide (mg•g^{-1} f.w.)	
	Lentinan	GGF
Aphyllophorales		
Ramaria botrytis	**0**	**trace**
Glifora frondosa	**0.9 ± 0.6**	**2.4 ± 1.0**
Sarcodon aspratus	**2.2 ± 0.8**	—
Meripilus giganteus	**1.0 ± 0.2**	**0.8 ± 0.04**
Agaricales		
Panellus serotinus	**0.9 ± 0.2**	**7.3 ± 1.2**
Lactarius hatsudake	**2.3 ± 0.8**	**4.1 ± 1.0**
Lentinus edodes	**3.4 ± 0.4**	**3.0 ± 0.5**
Hygrophorus russula	**0.8 ± 0.2**	—
Agaricus blazei	**0**	**trace**
Agaricus bisporus	**0**	**trace**
Flammulina velutipes	**2.5 ± 0.2**	**6.6 ± 2.1**

The contents of GGF in various fresh mushrooms

The contents of GGF or GGF-like polysaccharides in mushrooms were calculated by inhibition assay of ELISA. As shown in Table III-1 right column, the content of GGF in *G. frondosa* was 2.4 $mg \cdot g^{-1}$ f.w. Among the other mushrooms tested, GGF-like polysaccharide contents in *P. serotinus*, *F. velutipes*, *L. hatsudake* and *L. edodes* were 7.3, 6.6, 4.1 and 3.0 $mg \cdot g^{-1}$ f.w., respectively, and these contents were more than that in *G. frondosa*. In contrast, that in *M. giganteus* was less than that in *G. frondosa*, this being 0.8 $mg \cdot g^{-1}$ f.w. And, the GGF-like polysaccharides were scarcely detected in *A. brazei*, *A. bisporus* and *R. bortryis*.

The changes in the contents of lentinan during the growth of L. edodes

The *L. edodes* fruiting bodies were harvested at three different growth stages (Figure III-1), that is, the fruiting bodies were harvested at early formation period of fruiting body (Stage I), at middle stage of formation period (Stage II; the cap opened moderately) and at last stage of formation period (Stage III; the cap opened completely). The average fruiting body weight of *L. edodes* at Stage I was 9.1 ± 1.4 g, and they were 17.0 ± 2.4 g and 18.1 ± 3.3 g at Stage II and III, respectively. As shown in Table III-2, the lentinan contents were 9.3, 18.0 and 15.9 mg in a *L. edodes* fruiting body at Stage I, II and III, respectively.



Stage I **Stage II** **Stage III**

Growth stage

Figure III-1. Appearance of *L. edodes* during growth.

Table III-2. The changes in lentinan contents during growth of *L. edodes* fruiting bodies. The fruiting bodies as shown in Figure III-1 were analyzed. Student's *t*-test: **P* < 0.001 between Stage I and II, ***P* < 0.05 between Stage II and III.

Growth stage	Lentinan contents (mg• mushroom^{-1})	
Stage I	9.3 ± 1.1	*
Stage II	18.0 ± 1.3	**
Stage III	15.9 ± 0.3	**

The changes in the contents of GGF during the growth of G. frondosa

The *G. frondosa* fruiting bodies were harvested at four different growth stages (Figure III-2). The cap of *G. frondosa* at Stage I and II remained closing, and then it

opened moderately at Stage III, completely at Stage IV. The average weight of fruiting bodies increased to approximately 200 g at Stage IV. *G. frondosa* was commonly harvested as a commercial product at Stage IV. The GGF content was 2.3 mg in a *G. frondosa* fruiting body at Stage I, and they were 3.6, 4.8 and 2.7 mg in a fruiting body at Stage II, III and IV, respectively (Table III-3).

Stage I **Stage II** **Stage III** **Stage IV**

Growth stage

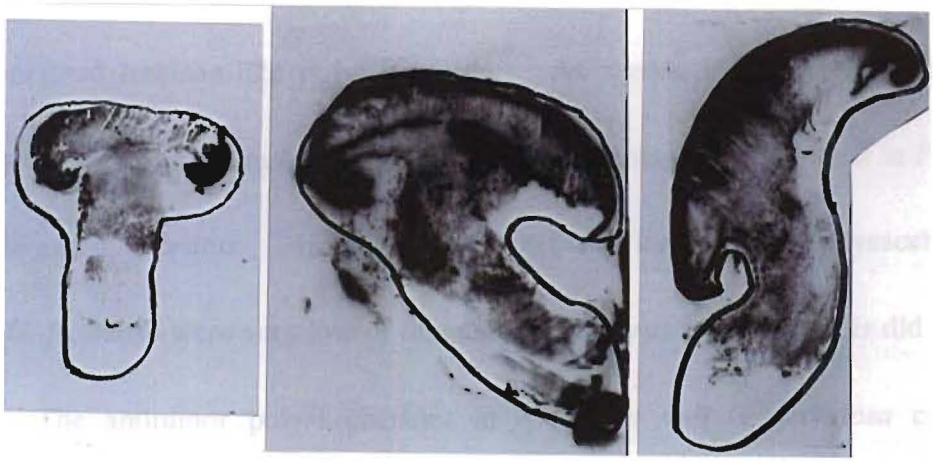
Figure III-2. Appearance of *G. frondosa* during growth.

Table III-3. The changes in GGF contents during growth of *G. frondosa* fruiting bodies. The fruiting bodies as shown in Figure III-2 were analyzed. Student's *t*-test: *$P < 0.05$ between Stage III and IV.

Growth stage	GGF contents (mg•mushroom^{-1})	
Stage I	**2.3 ± 0.3**	
Stage II	**3.6 ± 0.5**	
Stage III	**4.8 ± 1.4**	*
Stage IV	**2.7 ± 0.3**	*

Localization of lentinan in L. edodes tissue

To elucidate the locating site of lentinan in the fruiting body tissue of *L. edodes*, the author performed tissue prints that were immunostained for localizing lentinan by immunohistochemical analysis. Tissue prints of *L. edodes* at different growth stages immunostained by the anti-lentinan antibodies were shown in Figure III-3. Lentinan was detected in the tissue at early formation period of fruiting body (Stage I), and it mainly located in the cap. Lentinan accumulated markedly in the cap during growth at from Stage I to Stage II, and it was detected at low level in the stipe site of fruiting body. And then, lentinan decreased during growth at from Stage II to Stage III of *L. edodes* tissue.



Stage I **Stage II** **Stage III**

Growth stage

Figure III-3. Localization of lentinan in *L. edodes* fruiting bodies by western blotting analysis using anti-lentinan antibodies. The fruiting bodies as shown in Figure III-1 were analyzed.

III-4 DISCUSSION

The anti-lentinan and anti-GGF antibodies were raised by immunizaition, and the inhibition assay of ELISA was established for the quantitative determination of lentinan and GGF, respectively, in Chapter II. Many investigators had reported on the antitumor polysaccharides in several mushrooms. Most of these antitumor polysaccharides were β-1, 3 and β-1, 6 glucan with glucose residues side chains as well as lentinan and GGF. In this study, the author attempted to apply the inhibition assay of ELISA for the screening test of lentinan-like and GGF-like polysaccharides as antitumor polysaccharides in several mushrooms. In this chapter, the mushroom containing lentinan-like polysaccharide at high level contained also GGF-like polysaccharide at relatively high level. However, *G. frondosa* and *P. serotinus*, which contained GGF-like polysaccharide at relatively high level, scarcely contained lentinan-like polysaccharide. As shown in Table III-1, the lentinan content was the highest level in *L. edodes*, in turn its contents were high level in *F. velutipes*, *L. hatsudake* and *S. aspratus*. However, the contents of lentinan-like polysaccharide in *A. brazei* and *G. frondosa* were very low or less, and *A. bisporus* and *R. botrytis* did not contain it at all. The antitumor polysaccharides in *A. brazei* and *G. frondosa* contain β-1, 3-D-glucan main chains as like lentinan, this seems that structure and molecular weight of antitumor glucan are different among these glucans. On the other hand, the contents of GGF-like polysaccharide were higher level in *P. serotinus*, *F. velutipes*, *L. hatsudake* and *L. edodes* than GGF content in *G. frondosa*. However, GGF-like polysaccharides were scarcely detected in the extract from *A. brazei*, *A. bisporus* and *R. botrytis*. *R. botrytis*, *G.*

frondosa, *M. giganteus* and *S. aspratus* are classified into the order of Aphyllophorales, and the others into the order of Agaricales. Thus, there were no relationship between the antitumor polysaccharide and the order of tested mushroom. Although *F. velitipes* and *P. serotinus* belong to the family of Tricholomataceae, the content of lentinan-like polysaccharide in the former was more than that in the latter significantly. And also, although *G. frondosa* and *M. giganteus* belong to the family of Polyporaceae, the content of GGF-like polysaccharide in the latter was significantly less than that in the former. These results also suggested that the content of antitumor polysaccharide do not relate to the family of mushroom. In this chapter, it was found that *F. velutipes* contained lentinan-like and GGF-like polysaccharide at relatively high level. It has been reported that *F. velutipes* of those mushrooms showed significant antitumor activity (Ikekawa *et al.*, 1982 and Ohkuma *et al.*, 1982). These data suggest that the anti-lentinan and anti-GGF antibodies prepared in Chapter II may recognize the β-glucan having antitumor activity in the extract from mushrooms tested in this chapter. Thus, it is suggested that the immunochemical method can be possible to apply as screening test to the determination of antitumor polysaccharides in mushrooms. However, despite it is reported that the hot water extract of *A. brazei* contained β-1, 6 branched β-1, 3-glucan, such as lentinan, possessing antitumor activity (Mizuno *et al.*, 1990a) and that the extract of *A. bisporus* have significant antitumor activity (Nanba and Kuroda, 1988), *A. brazei* and *A. bisporus* did not contain both lentinan-like and GGF-like polysaccharides in this study. These results show that the structure and molecular weight of antitumor glucan may be different in the family of mushroom.

In this chapter, the contents of lentinan and GGF were determined in the fruiting bodies at different growth stage of fruiting bodies of *L. edodes* and *G. frondosa*, respectively, by the inhibition assay of ELISA. As shown in Figures III-1 and 2, both mushrooms developed significantly, and the average weight of both mushroom fruiting bodies increased during growth. The lentinan content was 9.3 $mg \bullet g^{-1}$ f.w. in one *L. edodes* fruiting body at Stage I during growth as shown in Table III-2. The contents of lentinan per *L. edodes* fruiting body increased during growth at from Stage I to Stage II, became to 18.0 mg at Stage II. However, it decreased to 15.9 mg in a fruiting body during growth at from Stage II to Stage III. These results suggested that the lentinan was synthesized in *L. edodes* during growth at Stage I to Stage II of fruiting body, but it was degraded during growth at from Stage II to Stage III. Moreover, in this chapter, it was elucidated that lentinan located in the site of cap of fruiting body mainly by immunohistochemical analysis (Figure III-3). As shown in Figure III-3, lentinan has been already synthesized during growth at from Stage I to Stage II, and lentinan markedly accumulated at Stage II. However, lentinan content decreased during growth at from Stage II to Stage III, suggesting that these results coincided with the results in Table III-2. Thus, it is beneficial, from the view of food functional point, that *L. edodes* as a functional food is harvested at middle formation period of fruiting body (Stage II; at formation period when the cap opens moderately). Although the GGF content in one *G. frondosa* fruiting body increased during growth at from Stage I to Stage III of fruiting body, and its content decreased during growth at from Stage III to Stage IV (Table III-3). It is important to confirm how much the antitumor polysaccharide is contained in

the fruiting body, when the mushroom is harvested. In this chapter, it was clarified that their contents were not maintained during the growth of the mushroom.

On the basis of these results, it was demonstrated that the inhibition assay of ELISA using the prepared antibodies in this study could be applied to evaluate the quality of the mushroom as a functional food. It was also confirmed that the prepared antibodies against antitumor polysaccharides could be utilized for the detecting the location site of antitumor polysaccharides in tissue of mushrooms.

IV BEHAVIOR OF ANTITUMOR POLYSACCHARIDES IN THE MUSHROOMS DURING STORAGE

IV – 1 INTRODUCTION

Chihara *et al* (1969, 1970) had reported that lentinan content was 15.5 mg per 100 g of fresh fruiting bodies of *L. edodes*. Nanba *et al* (1987a) reported that GGF was contained less than 16 mg per 100g of fresh fruiting bodies of *G. frondosa*.

It is important that these antitumor polysaccharides are maintained in the mushrooms during storage after harvest. However, the relationship between quality reduction of mushrooms and the decreases in antitumor polysaccharides contents in the mushroom have not yet been reported. Thus, it has remained obscure whether lentinan and GGF contents change during storage of the mushrooms. In this chapter, the author attempted to examine the changes in lentinan and GGF contents in *L. edodes* and *G. frondosa*, respectively, during storage at different temperatures. Furthermore, the author attempted to clarify a relationship between lentinan degradation and glucanase activity during storage of *L. edodes*.

Nanba *et al* (1987b, c) reported that *L. edodes* extract showed antitumor activity, and they suggested that this activity might be dependent on lentinan, an antitumor polysaccharide, in the extract. However, it was not clear whether the difference of antitumor activities of *L. edodes* stored under different storage conditions was related to the change in lentinan contents during storage. In this chapter, the author investigated the difference in the

immunomodulating effects of extracts from *L. edodes* stored at different temperature on cytokines productions from murine peritoneal macrophages. Recent studies have demonstrated that murine macrophages stimulated with antitumor polysaccharide to release cytokines such as IL-1 (interlueikin-1), IL-6, TNF-α and NO (Adachi *et al.*, 1994, Sakurai *et al.*, 1994, Okazaki *et al.*, 1995, Liu *et al.*, 1996). TNF-α and NO, especially, are focused as the cytokines that might play important roles in immune system of host implanted tumors. It has reported that lentinan induced TNF-α and NO from murine macrophages (Kerékgyártó *et al.*, 1996, Irinoda *et al.*, 1992). Therefore, the author examined the immunomodulating activity of *L. edodes* in TNF-α and NO productions as an indication of antitumor activity of the edible mushroom.

IV – 2 MATERIALS AND METHODS

Mushrooms

Lentinus 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 for 7 days, respectively. *Grifola frondosa*, commonly called "Maitake", was kindly provided by Yukiguni Maitake Co., Niigata, Japan. *G. frondosa* were packed in the pored polyethylene bags, and stored at 5 and 20°C for 7 days. 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 and GGF contents during storage of the mushrooms

Antibodies against lentinan and GGF were prepared according to the method described in Chapter II. Purified lentinan was kindly supplied by Ajinomoto Co (Japan). The authentic GGF was prepared as described in Chapter II. The crude lentinan and GGF fractions from the samples were prepared essentially according to Chihara *et al* (1970). The fresh mushroom fruiting bodies were homogenized with liquid nitrogen by Waring blender and lyophilized. The lyophilized powder sample (10 g) was extracted with 100 ml hot water for 10hr, and then the suspension was filtered to remove insoluble matter. The crude antitumor polysaccharide fractions were obtained by precipitation with an equal volume of ethanol to filtrate. The precipitate was centrifuged and lyophilized. The lentinan and GGF contents in the extract from the mushrooms were determined by the inhibition assay of ELISA.

Assay of polyphenol oxidase activity

Polyphenol oxidase (PPO) activity was determined by 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 ten-fold 0.05M phosphate buffer (pH 6.0). The suspension was centrifuged at 10,000 × 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 (15,000 × 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 phosphate buffer solution using a PD-10 column (1.6 × 5 cm, Amersham Pharmacia Biotech), then used as an enzyme solution. The reaction solution, consisting of 1.0 ml of McIlvaine buffer (pH 4.5) with 3.5 $mg \cdot ml^{-1}$ substrate and 40μl 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 one unit of enzyme activity.

Preparation of peritoneal macrophages

Peritoneal macrophages were isolated from female BALB/c mice, 6 to 8 weeks old (Japan SLC, Shizuoka, Japan). The mice had been injected intraperitoneally with 2 ml of thioglycolate medium 3 days prior to peritoneal lavage with 10 ml of serum-free RPMI 1640 from mice. 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 adhesion to plates for 2hr at 37°C and 5 % CO_2. After incubation, the cultures were washed to remove the nonadherent cells. Then fresh RPMI 1640 medium containing 10 % fetal bovine serum (FBS) and various concentrations of samples were added to the plates, and cultured for 24hr at 37°C and 5% CO_2. After incubation, the supernatant of cultures was collected and used for nitrite assay, and then stored at -80°C until TNF assay. In order to confirm the direct effect of the extract on the TNF-α production, the mice were individually injected with 10 mg from each of four extracts. One extract was prepared from fresh *L. edodes*; the others had been from stored *L. edodes* for 7 days, one at 1°C, one at 5°C, and one at 20°C. In addition, the extracts were dissolved in physiological saline (10 $mg \cdot ml^{-1}$) and injected into

mice i.p. one day before macrophages were gathered. Peritoneal cells were harvested from mice treated with physiological saline as control subjects.

Measurement of TNF-α amounts by L929 cells

TNF-α was measured by means of a cytolytic assay with actinomycin D-treated L929 cells (Takada *et al.*, 1994), using murine rTNF-α as the standard. L929 cells (2×10^5 cells•ml^{-1}) were plated in 96-well microplates in RPMI 1640 medium that included 5 % FBS and cultured for 2 hours. Fifty μl of supernatant samples obtained from macrophages stimulated with extract of *L. edodes* or authentic lentinan and 50μl samples of actinomycin D (4μg•ml^{-1}) were added to the culture plates; they were then cultured for 20 hr 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.) at 570 nm (main) and 630 nm (reference).

Nitrite assay

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 a sodium nitrite as a standard.

Assay of β-glucanase activity

All operations for the preparation of enzyme extracts were performed according to the procedure described by Ohga (1992). The fruiting body of *L. edodes* was added to a cold, ten-fold 10 mM acetate-acetic acid buffer solution (pH 4.2) and homogenized in a Waring blender. The suspension was centrifuged at 10,000 × 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 hr, the precipitate was collected by centrifugation at 15,000 × g for 20 min, redissolved in a 1 mM acetate acetic acid buffer solution (pH 4.2) then centrifuged to removed insoluble material at 15,000 × 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* fruiting body as substrate. Assay was performed in a 30 mM acetate acetic acid buffer solution (pH 4.2) with 0.2 $mg \cdot ml^{-1}$ substrate at 40°C for 30 min, and the mount of the released reducing sugar was determined by the Somogyi-Nelson method. One unit of activity was defined as 1 μmol of reducing sugar released (as glucose equivalents) per minute.

IV-3 RESULTS

Decrease in the lentinan contents of L. edodes during storage

The appearance of *L. edodes* fruiting body was shown in Figure IV-1. Although it did not changed during storage at 1 and 5°C, it browned rapidly at 20°C. And also, PPO activity, which associated with the browning of *L. edodes* (Minamide *et al.*, 1980), increased rapidly during storage at 20°C, but did not change at low temperatures (1 and 5°C) (Figure IV-2).

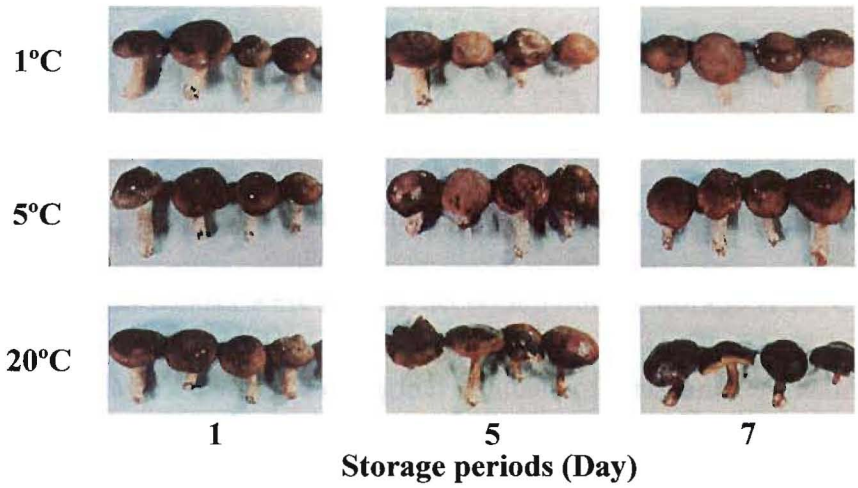


Figure IV-1. Appearance of *L. edodes* during storage.

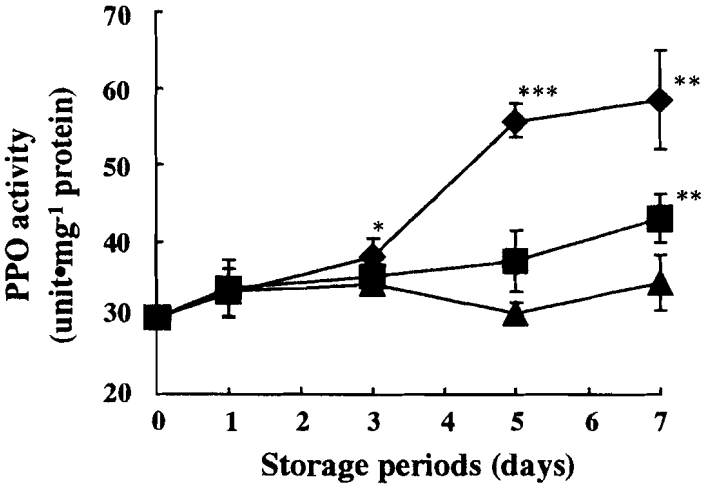


Figure IV-2. Changes in PPO activity of *L. edodes* during storage. *L. edodes* fruiting bodies were stored at 1°C; (▲), 5°C; (■) and 20°C; (◆). Student's *t*-test: **P* < 0.05, ***P* < 0.02 and * *P* < 0.001 against 0 day.**

Figure IV-3 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* fruiting body contained 1.1 $mg \cdot g^{-1}$ fw 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* were rapidly decreased during storage at 20°C, and reach to 0.3 $mg \cdot g^{-1}$ fw after 7 days. While the original contents of lentinan were 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 0.8 $mg \cdot g^{-1}$ fw after 7 days at 5°C. These results indicated that the lentinan content in the fruiting body decreased rapidly during storage at 20°C and that low temperature storage was effective to maintain lentinan contents in *L. edodes*.

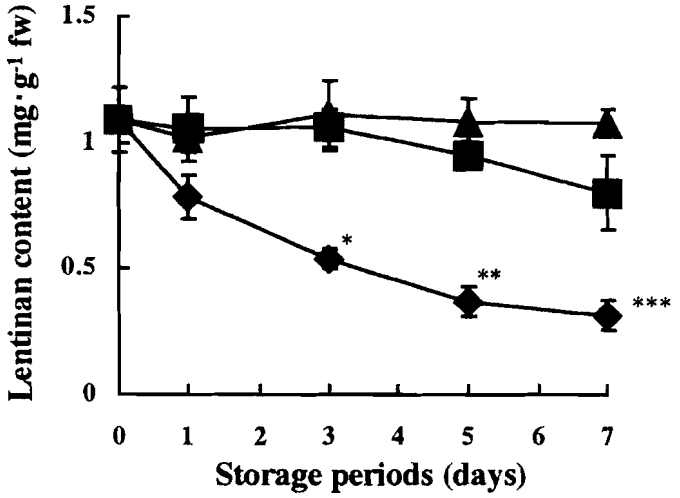


Figure IV-3. Changes in lentinan content of *L. edodes* during storage. *L. edodes* fruiting bodies were stored at 1°C; (▲), 5°C; (■) and 20°C; (◆). Student's *t*-test: **P* < 0.02, ***P* < 0.01, and ****P* < 0.005 against 0 day.

Difference in the effects of extracts from stored L. edodes on TNF-α production

We investigated the difference in effects of hot water extract, which contained lentinan fraction, from *L. edodes* stored at different temperatures on TNF-α production from mouse peritoneal macrophages by using L929 cells. As shown in Figure IV-4, the amounts of TNF-α released from macrophages stimulated with extracts from *L. edodes* stored at 1 and 5°C for 7 days were maintained 71.3 % and 80.1 %, respectively, compared with the initial level of TNF-α productions *in vitro* from macrophages stimulated with fresh *L. edodes* extract. However, the amount released from macrophages stimulated with the extracts from *L. edodes* stored at 20°C for 3 days and 7 days drastically reduced to 36.6 % and 20.5 %, respectively, compared with the initial level.

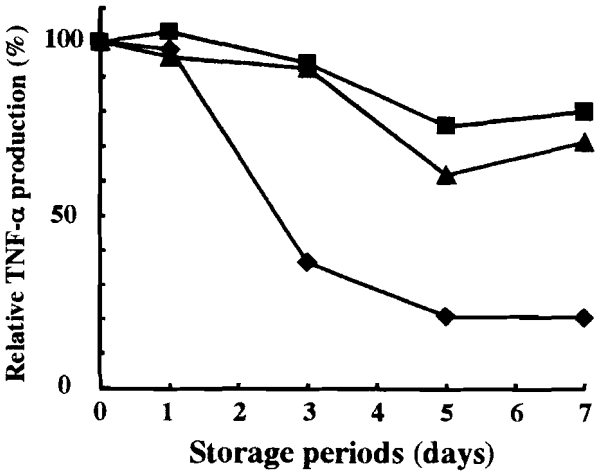


Figure IV-4. Changes in relative production of TNF-α from mice macrophages *in vitro* stimulated with stored *L. edodes* extracts. *L. edodes* fruiting bodies were stored at 1°C; (▲), 5°C; (■) and 20°C; (◆) for 7 days. TNF-α production from macrophages stimulated fresh *L. edodes* (0 day) extract were arbitrarily set at 100%.

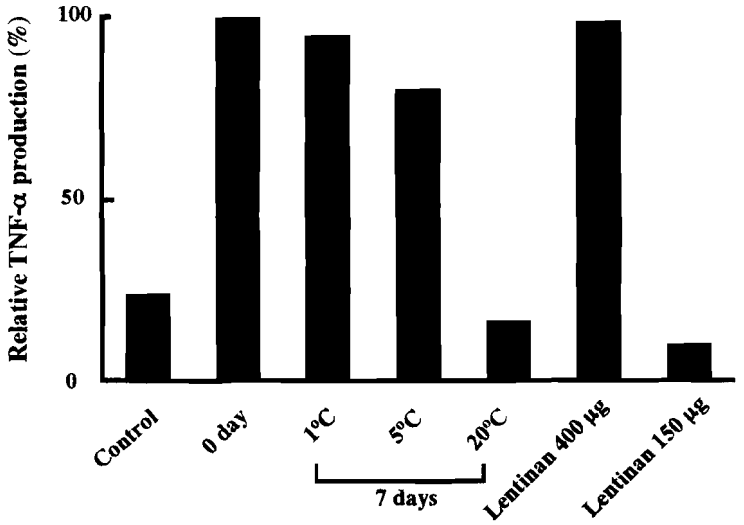


Figure IV-5. Effect of extracts from stored *L. edodes* on TNF-α production *in vivo* from macrophages. *L. edodes* fruiting bodies were stored at 1°C, 5°C and 20°C for 7 days. TNF-α production from macrophages stimulated fresh *L. edodes* (0 day) extract were arbitrarily set at 100%.

Figure IV-5 shows the effects of the extracts from stored *L. edodes* on TNF-α production *in vivo*. The amounts of TNF-α released from macrophages stimulated with the extract from *L. edodes* stored at low temperatures (1 and 5°C) did not differ drastically compared to fresh *L. edodes* extract. However, TNF-α production from macrophages stimulated with the extract from *L. edodes* stored at 20°C was significantly decreased to 16.9 %; it was the same tendency as *in vitro*, as shown in Figure IV-4. These results indicated that the effects of *L. edodes* extracts on TNF-α production were maintained in the mushroom stored at low temperatures (1 and 5°C).

Lentinan content was approximately 40 $\mu g \cdot mg^{-1}$ in extract of fresh *L. edodes*, and this content did not drastically change during low temperature storage. However, the content decreased to 15 $\mu g \cdot mg^{-1}$ in an extract of the mushroom during storage at 20°C for 7 days. In order to confirm whether lentinan contents in *L. edodes* extract really reflected TNF-α production, the author examined the effect of authentic lentinan on TNF-α production from macrophages. When animals were injected with an equal content of authentic purified lentinan (400 μg and 150 μg) to lentinan content in the extracts (10 mg) from *L. edodes* which were fresh and stored at 20°C for 7 days, TNF-α was released at the same level from macrophages stimulated with each extract (Figures IV-4 and 5).

Difference in the effects of extracts from stored L. edodes on NO production

NO production *in vitro* from macrophages stimulated with the extract from fresh *L. edodes* was 7.4 ± 1.6 μM (Figure IV-6). In the extracts from *L. edodes* stored for 7 days,

NO production was not significantly differ compared to the fresh *L. edodes* extract under storage temperatures at 1 and 5°C, however its production drastically decreased in the extract from *L. edodes* stored at 20°C. NO production from macrophages stimulated with the extracts from *L. edodes* stored at 1 and 5°C was 5.9 ± 0.4 and 5.9 ± 1.1 μM, respectively. However, NO production (0.4 ± 0.3 μM) from macrophages stimulated with the extract from *L. edodes* stored at 20°C did not increase at all compared with the production (0.6 ± 0.5 μM) from non-stimulated macrophages. These data demonstrated that the effect of extract from *L. edodes* stored at 20°C on NO production was a decrease, compared to the effect of fresh *L. edodes* extract similar to that on TNF-α production. And also, macrophages stimulated with 400 μg of lentinan, corresponding to the lentinan content in fresh *L. edodes* extract, released 7.9 ± 1.5 μM of NO. NO production from macrophages stimulated with 150 μg of lentinan, corresponding to the extract during storage of *L. edodes* for 7 days at 20°C,

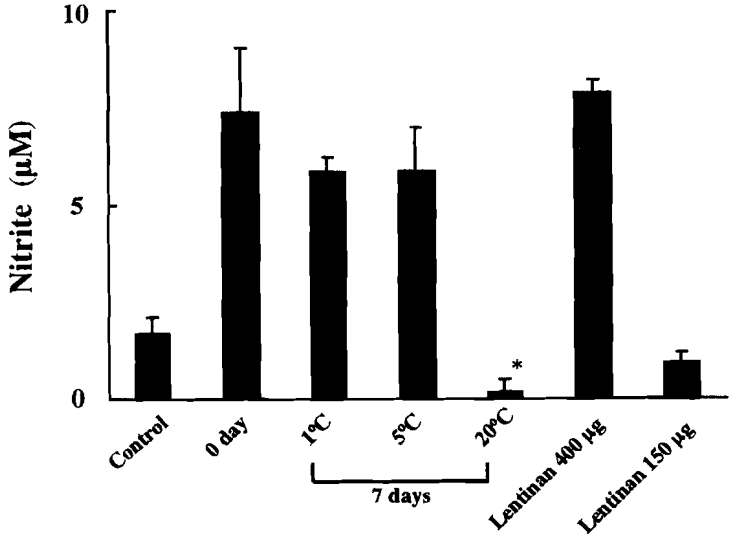


Figure IV-6. Effect of extracts from stored *L. edodes* on NO production from macrophages. *L. edodes* fruiting bodies were stored at 1°C, 5°C and 20°C for 7 days. All data points are mean + SD (n = 3). Student's *t*-test: *$P < 0.005$ against 0 day.

decreased significantly to 1.0 ± 0.3 μM, and this amount of NO production was almost the same as the amount produced from non-stimulated macrophages.

Changes in β-glucanase activity in L. edodes during storage

The changes in β-glucanase activity during storage of *L. edodes* were measured by the Gong's method using lentinan as a substrate to make clear the degradation mechanism of lentinan. The β-glucanase activity was at a very low level (0.27 $U \cdot mg^{-1}$) 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 $U \cdot mg^{-1}$ 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 $U \cdot mg^{-1}$ after 3 days (Figure IV-7).

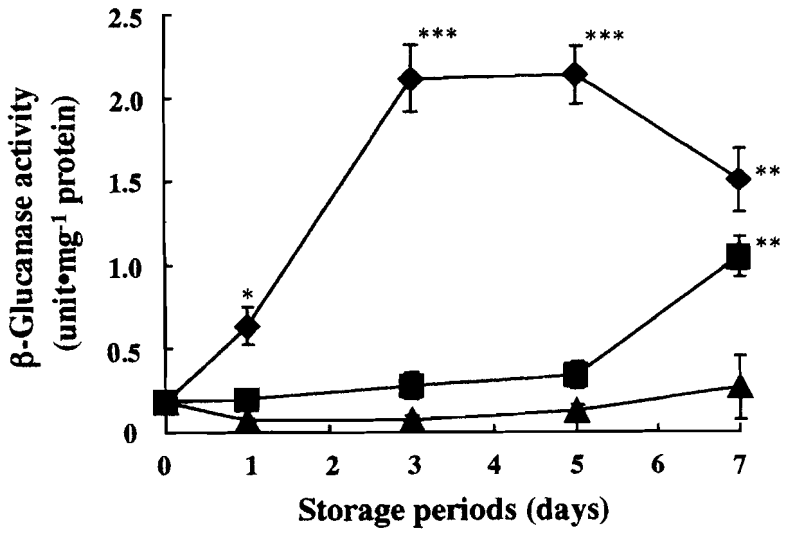


Figure IV-7. Changes in β-glucanase activity during storage of *L. edodes*. *L. edodes* fruiting bodies were stored at 1°C; (▲), 5°C; (■) and 20°C; (◆). Student's *t*-test: *$P < 0.025$, **$P < 0.005$ and ***$P < 0.001$ against 0 day.

Decrease in the GGF contents of G. frondosa during storage

The changes in the contents of GGF in *G. frondosa* during storage at 5 and 20°C was calculated based on the results of the inhibition assay of ELISA. The appearance of *G. frondosa* fruiting body browned more at 20°C than at 5°C during storage (Figure IV-8).

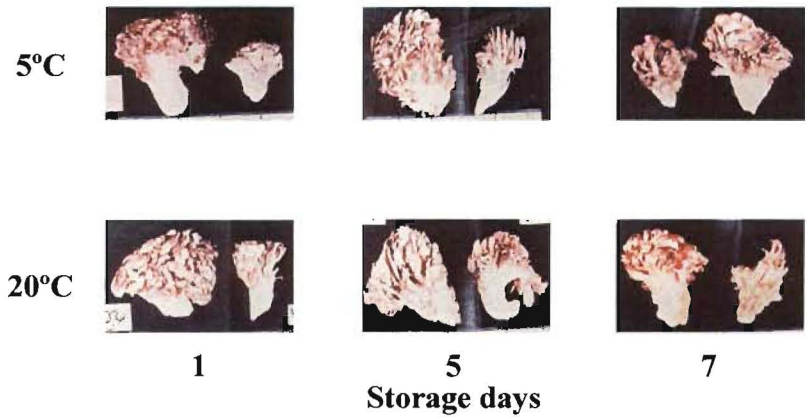


Figure IV-8. Appearance of *G. frondosa* during storage

As shown in Figure IV-9, the content of GGF immediately after harvest was at its highest, with a 24.7 $\mu g \cdot g^{-1}$ f.w. The GGF content decreased to 11.4 $\mu g \cdot g^{-1}$ f.w. during storage at 20°C for 7 days. In contrast, the content did not change drastically during storage at 5°C for 7 days. The weight of *G. frondosa* (201.7 ± 6.9 g) decreased significantly to 185.8 ± 8.7 g during storage for 7 days at 20°C, and only to 196.9 ± 2.2 g at 5°C. The rate of weight loss at 7 days during storage at 20°C and 5°C were approximately 8 % and 3 %, respectively, as compared with a fresh weight of *G. frondosa* fruiting body immediately after harvest.

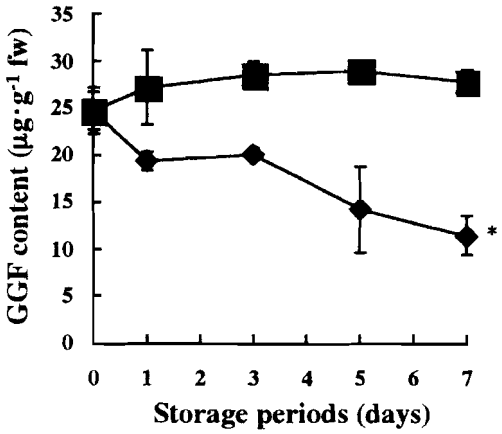


Figure IV-9. Changes in GGF content of *G.frondosa* during storage. *G. frondosa* fruiting bodies were stored at 5°C; (■) and 20°C; (◆). Student's *t*-test: *$P < 0.02$ against 0 day.

IV-4 DISCUSSION

Edible mushrooms have become attractive as healthful foods (functional food); they are known to contain some kinds of immunomodulating polysaccharides. Many investigators have reported on the antitumor activities of some mushrooms containing antitumor polysaccharides, such as *L. edodes* (Nanba *et al.*, 1987b, c, Sasaki and Takasuka, 1976), *Schizophyllum commune* (Komatsu *et al.*, 1969), *G. frondosa* (Nanba *et al.*, 1987a), *Agaricus blazei* (Kawagishi *et al.*, 1986), and *Corioulous versicolor* (Tsukagoshi *et al.*, 1984). It is important that the contents of these antitumor polysaccharides are kept during storage of the mushrooms. However, it has not been clear whether the contents of these antitumor polysaccharides change with the reduction of freshness of the mushroom fruiting bodies during storage, since the exact and simple method had not been established for the determination of a polysaccharide. The author confirmed that immunochemical analysis

using the antibody against the targeting polysaccharide could be an effective method for the determination of an antitumor polysaccharide in the fruiting body of mushroom in Chapter II. In this chapter, the changes in the contents of antitumor polysaccharides, lentinan and GGF, in *L. edodes* and *G. frondosa* with reduction of freshness during storage were examined by the inhibition assay of ELISA as described in Chapter II.

The *L. edodes* and *G. frondosa* fruiting bodies kept their freshness under low temperature storage (1 and 5°C), but they were browning rapidly at 20°C (Figures IV-1 and 8). It has been reported that the quality reduction of mushrooms is closely related to their browning, and the browning of the fruiting body is caused by the increased PPO activity during storage (Minamide *et al.*, 1980 a, b, c; and Yamaguchi *et al.*, 1988). Thus, PPO activity was also examined as an index of the quality reduction during storage of *L. edodes*. As shown in Figure IV-2, although PPO activities changed little during storage of *L. edodes* at low temperatures, it increased significantly with the browning of the fruiting body at 20°C during storage. These results were consistent with the reports of Minamide *et al* (1980 a, b) and Gong *et al* (1993). Regarding to *G. frondosa*, it has been reported that the freshness of the fruiting body was extended longer at 5°C than 10, 20 and 30°C, and no drastic changes in the freshness were observed at 1 and 5°C (Yoshida *et al.*, 1991). Indeed, the weight loss of *G. frondosa* fruiting body was kept at low level (3 %) for 7 days and the browning of fruiting body did not appear during storage at 5°C, and its freshness was kept in this experiment. However, the weight loss increased significantly to 8 % during storage at 20°C for 7 days, and the fruiting body was browning rapidly. Figure IV-3 shows the time

course changes in lentinan contents in hot water extracts from *L. edodes* during storage at 1, 5, and 20°C. 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* were rapidly decreased during storage at 20°C for 7 days, while the initial level of lentinan was maintained during storage for 5 days at 5°C and for 7 days at 1°C. And also, the contents of GGF in *G. frondosa* was at its highest immediately after harvest, and did not change drastically during storage at 5°C for 7 days. It, however, decreased significantly during storage at 20°C (Figure IV-9). These results indicated that the contents of antitumor polysaccharides in the fruiting body decreased rapidly during storage at 20°C, and suggested that the low temperature storage was effective to maintain antitumor polysaccharides contents and the quality of the mushroom as a functional food.

Lentinan is known as a type of biological response modifier (Hamuro and Chihara, 1985). 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, Taguchi and Kaneko, 1986, Nanba and Kuroda, 1987b and Gergely *et al.*, 1988). And also, it has been reported that lentinan possessed an immunomodulating effect, which was seen in the activation of a variety of macrophage functions, e.g. interleukin-1 and superoxide anion production, phagocytosis, and cytotoxicity (Maeda *et al.*, 1971, Freunhauf *et al.*, 1982, Akiyama *et al.*, 1987, Ábel *et al.*, 1989, Herlyn *et al.*, 1983, Chihara *et al.*, 1987, Nanba *et al.*, 1987a and Ladányi *et al.*, 1993) in their antitumor activities. This antitumor function of macrophages may comprise two

mechanisms: cell to cell contact between macrophages and tumor cells, and the release of antitumor factors such as cytokines (TNF-α and interleukin etc.), eicosanoids, reactive oxygen metabolises, and nitrogen intermediates such as NO. Recently, it has been reported that macrophages secreted TNF-α through the stimulation of lentinan, both *in vitro* and *in vivo* (Kerékgyártó *et al.*, 1996) and the other antitumor polysaccharides (Okazaki *et al.*, 1995). TNF-α is recognized as the primary cytokine produced mainly by activated macrophages; it is an important host defense molecule that affects tumor cells (Carswell *et al.*, 1975). Hoffman *et al* (1993) observed that TNF-α was released from macrophages through a β-glucan mediated mechanism.

NO has also recently been identified as a mediator of the antitumor effect of macrophages. It has been reported that mouse macrophages stimulated with antitumor polysaccharides produced NO (Asai *et al.*, 1996), and also macrophages stimulated with lentinan released NO (Irinoda *et al.*, 1992). NO is a noxious, stable, free radical gas, and plays an important role in the functions of macrophages. Much attention has focused on the role of NO, which has shown itself to be an essential mediator of diverse functions, including immunoresponse, vasodilatation, neurotransmission, inhibition of platelet aggregation, and inflammation (Lowenstein and Synyder, 1992). NO was first recognized in mammalian physiology as a mediator of macrophage actions. The 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. These data seem to suggest

that enhancement of TNF-α and NO production through macrophages may reflect the antitumor activity of *L. edodes.*

In this chapter, the author investigated the difference in effect of the extract from *L. edodes* stored at different temperatures on TNF-α and NO productions from macrophages; these productions were indications of immunomodulating activity. Figures IV-4, 5 and 6 showed about the difference in the immunomodulating effects of stored *L. edodes* on cytokines producrions from murine peritoneal macrophages. The author ascertained that the effect of *L. edodes* extract on TNF-α production from mouse macrophages showed little difference between *L. edodes* stored at low temperatures and the fresh mushroom, although the cytokine production decreased significantly in *L. edodes* stored at 20°C (Figure IV-4). TNF-α production *in vivo* from macrophages stimulated with *L. edodes* extract showed the same tendency as *in vitro* (Figure IV-5). NO production was scarcely different in the *L. edodes* extract stored at low temperatures compared with the extract from the fresh mushroom immediately after harvesting. However, NO production drastically decreased in the extract from the mushroom stored at 20°C for 7 days (Figure IV-6). In order to clarify whether lentinan content influenced the cytokines productions from macrophages, we examined TNF-α and NO productions induced by purified lentinan. Macrophages stimulated with 400 μg of purified lentinan, corresponding to the lentinan content in 10 mg of extract from fresh *L. edodes*, produced a level of cytokines almost equal to that induced by *L. edodes* extract immediately after harvest. Also, amounts of the cytokines from macrophages stimulated with 150 μg of purified lentinan, corresponding to lentinan content

in the extract from *L. edodes* stored at 20°C for 7 days, were almost equal to the amounts of cytokines induced by the extract from *L. edodes* stored for 7 days at 20°C (Figures IV-5 and 6). These results suggested that lentinan itself in extract from the mushroom reflected the augmentation of the immunomodulating effect of *L. edodes* on TNF-α and NO production from macrophages.

In order to get a clue to the degradation of lentinan, the changes in β-glucanase activity during storage of *L. edodes* were measured (Figure IV-7). The β-glucanase activity was at a very low level (0.27 $U \cdot mg^{-1}$) immediately after harvest, and it was only slightly enhanced during storage at low temperatures (1 and 5°C). On the other hand, during storage at 20°C, the β-glucanase activity rapidly increased and peaked to 2.12 $U \cdot mg^{-1}$ after 3 days. These results suggest that the decrease in lentinan content during storage of *L. edodes* might be correlated closely to the increase in β-glucanase activity (Figures IV-3 and 7). Thus, it is inferred that this enzyme 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. Moreover, these all results seemed to indicate that the decrease in immunomodulating activity of *L. edodes* was closely correlated to decrease of lentinan content accompanied by enzymatic degradation during storage.

V DEGRADATION MECHANISM OF LENTINAN IN *LENTINUS EDODES* BY β-1, 3-GLUCANASE DURING STORAGE

V – 1 INTRODUCTION

In Chapter IV, it already demonstrated that lentinan in *Lentinus edodes* "Shiitake" extract was decreased during storage at 20°C, and that the increase in β-glucanase activity could correlate with the decrease in the lentinan contents during storage of *L. edodes*. Many investigators have reported about β-glucanases in some kinds of mushrooms. β-1, 3-Glucanases had been isolated from *Coprinus radians* "Kokiraratake" and *Coprinus macrorhizus* "Nenaganohitoyotake" (Kawai, 1970 and Kawai and Ishida, 1970). And also, β-1, 3-glucanases had been found in QM 806, Basidiomycetes species (Nelson *et al.*, 1969), and *Lentinus lepideus* "Matsuoji" (Takahashi *et al.*, 1978). Ohga (1993) had reported about the extracellular *endo*-β-1, 3-glucanase activity during fungal fruiting body formation of *L. edodes*. In this chapter, in order to elucidate the mechanism of the lentinan degradation in *L. edodes* during storage, the author isolated and purified β-1, 3-glucanase from *L. edodes*, and investigated some properties of it and its synthesis pattern during storage of *L. edodes*.

It was also described that the immunomodulating activity of *L. edodes* diminished with the decrease in the lentinan content in Chapter IV. However, it is not clarified as to the relationship between the decrease of immunomodulating activity and lentinan degradation by the β-glucanase. In this chapter, the author also attempted to investigate whether the

immunomodulating effects of lentinan on TNF-α and NO productions from mouse peritoneal macrophages changed when authentic lentinan was degraded by β-1, 3-glucanase purified from *L. edodes* fruiting body.

V – 2 MATERIALS AND METHODS

Materials

Lentinan was isolated from *L. edodes* according to the method of Chihara *et al* (1970). Laminarin, β-1, 3-glucan, from *Laminaria digesta* was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Pustulan, β-1, 6-glucan, 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).

Purification of β-glucanase from L. edodes

All operations for the preparation of enzyme extracts were performed at 4°C. *L. edodes* mushroom (500 g) stored for 4 days at 20°C was homogenized in 1 liter extraction buffer composed of 10 mM NaOAc solution (pH 4.2) containing 10 mM EDTA, 1 mM PMSF, and 20 mM β-mercaptoethanol. After homogenizing, insoluble material was removed by centrifugation, and the supernatant as a crude enzyme was fractionated with $(NH_4)_2SO_4$. Proteins precipitating in the 80% saturated $(NH_4)_2SO_4$ fraction were redissolved in the extraction buffer, and dialyzed against the 1mM NaOAc (pH 4.2) for 24 hr. Extracts were applied to DEAE Sepharose CL-6B column (5 cm × 80 cm, Amersham Pharmacia Biotech)

that had been equilibrated in 20 mM NaOAc (pH 6.0). After 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. The fractions which had 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 1mM NaOAc (pH 6.0). 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 anion-exchange chromatography a Mono-Q column HR 5/5 (5 mm × 50 mm) equilibrated with 20 mM NaOAc (pH 6.0) was used. A 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 with a flow rate of 1 ml•min^{-1}, and 1ml fractions were collected. Active fractions were further purified by FPLC gel-filtration chromatography with a Superose 12 column HR 10/30 (10 mm × 300 mm) equilibrated with 50 mM NaOAc (pH 5.0) containing 150 mM NaCl. 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 weights of the purified glucanases were estimated using aldolase (158 kDa), BSA (67 kDa), ovalbumin (43 kDa), ribonuclease (13.7 kDa) as standards.

Gel electrophoresis

The homogeneity and molecular masses of purified enzymes were determined by SDS-PAGE on 12% gels containing 5% stacker gel. Proteins were applied to the gels after treatment by the method of Laemmli (1970). Molecular weights were determined with the rainbow marker containing myosin (220 kDa), Phosphorylase b (97.4 kDa), Bovine serum albumin (66 kDa), Ovalbumin (46 kDa), Carbonic anhydrase (30 kDa) and Trypsin inhibitor (21.5 kDa) as standards. After running gels, the gels were stained with Coomassie Brilliant Blue R250.

Enzyme assay

β-Glucanase activity was routinely determined, in triplicate, using lentinan from *L. edodes* as substrate. Assays were performed in 30 mM NaOAc (pH 4.2) with 1 $mg \cdot ml^{-1}$ (w/v) substrate at 40°C for 30 min, and the amount of reducing sugar released determined by Somogyi-Nelson method (Nelson, 1944 and 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) and Lowry method (Lowry *et al.*, 1951) using BSA as a standard.

Enzymatic properties and kinetics

The pH optimum of *L. edodes* β-1, 3-glucanases were determined over the pH range 3.6 - 7.2 in 30 mM NaOAc (pH 3.6 - 5.6) and 10 mM sodium phosphate buffer (pH 6.0 - 7.2)

containing 200 $\mu g \cdot ml^{-1}$ of the *L. digitata* laminarin as a substrate. Kinetic determinations were performed at 40°C and at the respective optimum pH for the various enzymes. All substrates were used over the concentration range 0.05 - 1.0 $mg \cdot ml^{-1}$, and then data were analyzed by Michaelis-Menten kinetics. Substrate specificities of the purified β-glucanases were determined against a range of β-D-glucans at 1.0 $mg \cdot ml^{-1}$. Activities against all substrates were determined reductometrically as described above.

Enzymatic digestion of lentinan

Lentinan was digested by the purified enzyme solution. The purified glucanase solution (10 $units \cdot ml^{-1}$) was added to 10ml of 1$mg \cdot ml^{-1}$ lentinan solution in 30 mM NaOAc (pH 4.2) and then incubated for 15, 30 min, 4 hr and 48hr at 40°C. After inactivating the enzymes by heating for 10 min at 100°C, each lentinan content in incubation mixture with the β-glucanase was determined by ELISA inhibition assay as described in Chapter II. Then, the effects of the mixture with the purified glucanases on TNF-α and NO release from macrophages were determined as described in Chapter IV. Furthermore, to confirm the action modes of the purified β-glucanase isoenzymes, the incubation mixtures for 15min and 48 hr were applied to a Sephacryl S-300 HR column (1.6 × 90cm, Amersham Pharmacia Biotech) and eluted with a phosphate buffer (pH 7.2, 3ml fractions). Sugar contents in each fraction was monitored by the anthtone-H_2SO_4 method at 620nm using glucose as a standard. 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 L-3300 RI detector (Hitachi, Japan)

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

For preparation of antibody to purified *L. edodes* β-1, 3-glucanase, only the major protein band (Mw 72,000) on SDS-PAGE was extracted from the gel and used as the antigen. The β-1, 3-glucanase (0.5 mg) were dissolved in 1 ml of PBS (pH 7.2). This solution was emulsified with an equal volume of Freund's complete adjuvant. The emulsions were injected subcutaneously at 10 different sites in the back of the rabbits, individually. Half volume of a β-1, 3-glucanase in first dosage was boosted again two weeks after first injection. After the boost, the blood was collected to obtain anti-β-1, 3-glucanase sera several times every one week. The sera were stored at -80°C until use. After measurement of these antibodies titer, the rabbits were exsanguinated. Then IgG fraction of antisera was prepared by Protein G column (0.5 × 10 cm, Amersham Pharmacia Biotech).

Western blotting analysis

Proteins were separated by SDS-PAGE as described above and transferred to PVDF membranes (Amersham Pharmacia Biotech) under semi-dry transfer conditions. The membranes were blocked in TPBS containing 20 % skim milk overnight. The membranes were washed with TPBS and then incubated with rabbit antibodies IgG in TPBS containing 20 % skim milk for 1 hr at room temperature. Following incubation with the primary

antisera, the membranes were washed four times with TPBS (5 min / wash). Then the membranes were incubated with HRP conjugated goat anti rabbit IgG secondary antibody for 30 min at room temperature at dilution of 1:2,000. The membranes were washed five times with TPBS. Detection was performed by enhanced chemiluminescence according to manufacturer's specifications (Amersham Pharmacia Biotech).

Measurement of immunomodulating effects of lentinan degradation product

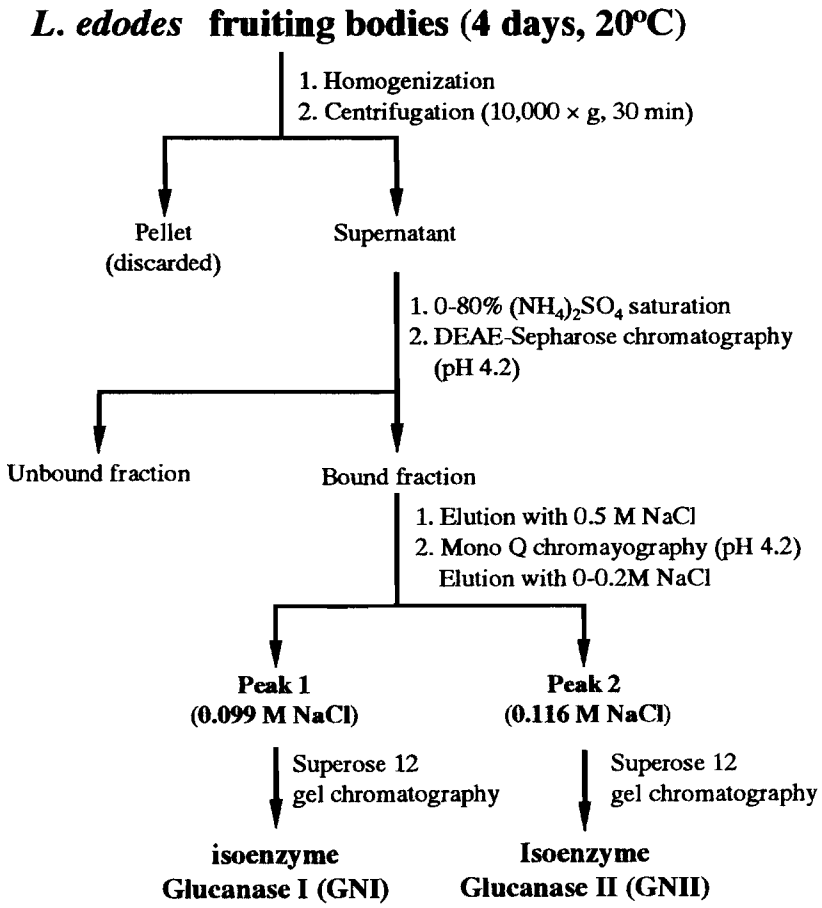
Lentinan was incubated with purified enzyme solution prepared as substantial β-glucanase (10 units•ml^{-1}) for 0.25 to 48 hr at 40°C (pH 4.2). After inactivation at 100°C for 10 min, lentinan content in the incubation mixture was measured by inhibition assay of ELISA. Moreover, the effect of the mixture on TNF-α and NO productions from mouse peritoneal macrophages was investigated as described in Chapter IV.

V-3 RESULTS

Enzyme purification

In order to investigate the property of β-glucanase that seemed to concern with the decrease of lentinan, these enzymes were isolated and purified. The purification factors, yields and specific activities of two β-glucanase isoenzymes were summarized in Table V-1. Selected chromatographic profiles were presented in Figures V-1 and 2, and SDS-PAGE of the purified glucanases is shown in Figure V-3. The elution pattern on anion-exchange (Mono-Q) column showed two peaks that hydrolyzed lentinan, designated GNI and II (Figure

V-1). Fractions eluted from the Mono-Q column with glucanase activity were further chromatographed on a Superose 12 column. The elution patterns on the gel column of GNI and II revealed single protein peaks respectively, corresponding to fraction that showed glucanase activity (Figure V-2). The purification factors of two β-glucanase isoenzymes were 93.8 and 47.6, and yields were 16.9 % and 7.7 %, respectively (Table V-1).



Scheme V. Summary of procedures used to purified β-glucanase isozymes GNI and GNII from *L. edodes* fruting bodies.

Table V-1. Summary of purification and yields of β-glucanases from *L. edodes*.

	Total Protein (mg)	Total activity (units)	Specific activity (unit•mg^{-1})	Yield (%)	Purification factor
Crude	3838.1	12842.3	3.3	100.0	1.0
$(NH_4)_2SO_4$	1049.3	11494.0	11.0	89.5	3.3
DEAE	352.7	6771.8	19.2	52.7	5.7
GN I					
MonoQ	11.2	2926.6	261.3	22.8	78.1
Superose12	6.9	2164.5	313.7	16.9	93.8
GN II					
MonoQ	14.7	1968.3	133.9	15.3	40.0
Superose12	6.2	988.3	159.4	7.7	47.6

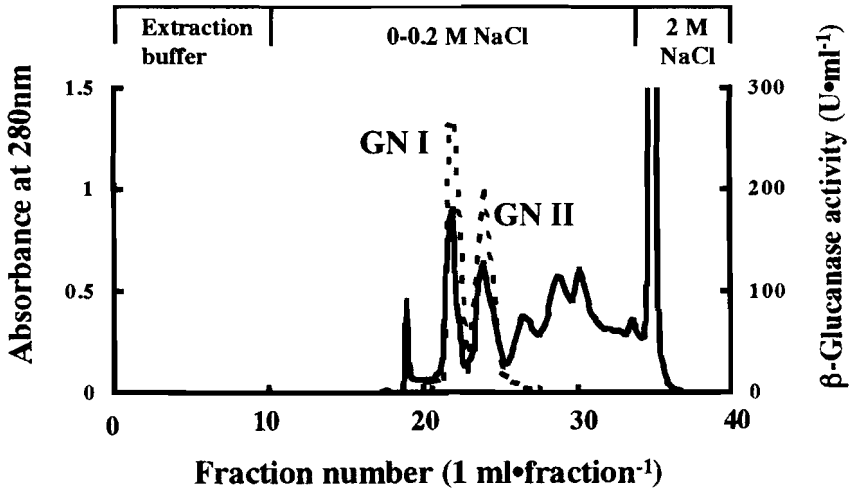


Figure V-1. Anion-exchange chromatogram of β-glucanases by FPLC. The fraction of *L. edodes* extract eluted with 0.5M NaCl on DEAE-Sepharose was applied to MonoQ column. Fractions (1 ml) were assayed for β-glucanase activity; (····) and protein (A_{280}); (—).

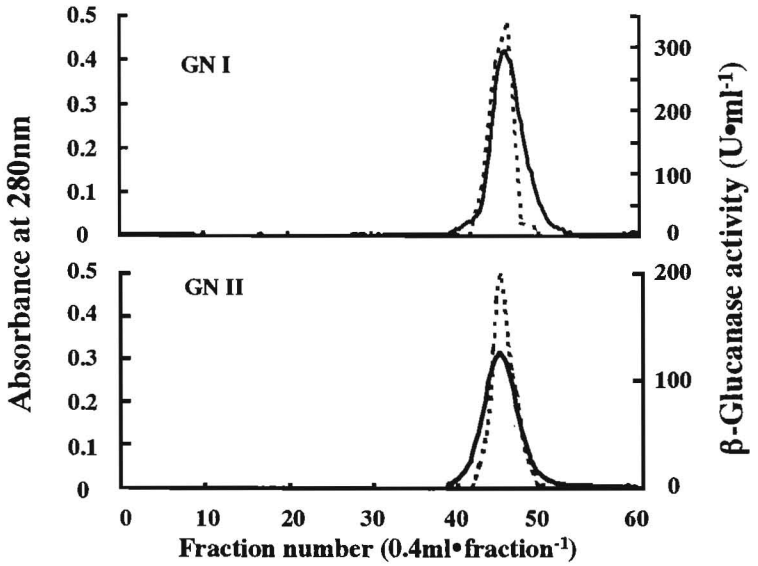


Figure V-2. Gel filtration chromatogram of β-glucanases by FPLC. GNI and GNII from the MonoQ column were applied to a Superose 12 column. Fractions (0.4 ml) were assayed for β-glucanase activity; (····) and protein (A_{280}); (—).

Properties of the purified glucanases

Analysis of each glucanase by SDS-PAGE showed in Figure V- 3. The molecular masses of GNI and II were estimated to be 72.9 and 74.7 kDa, respectively.

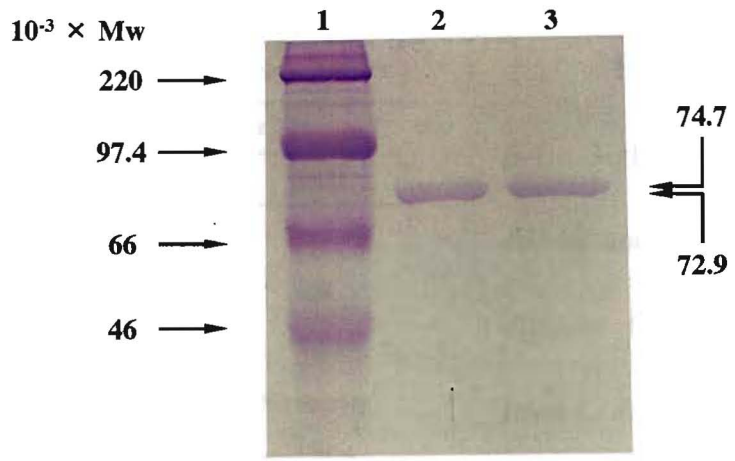


Figure V-3. SDS-PAGE of the purified *L. edodes* β-glucanases. Lane 1; molecular mass markers, lane 2; purified GN I, lane 3; GN II.

And also, single peak was obtained on the chromatogram by gel filtration of the each GN, and the molecular weights of both GNs were about 70 kDa (Figure V-2). These results suggested that two purified glucanases were monomeric protein.

The two purified *L. edodes* glucanase obeys linear Michaelis-Menten kinetics over the concentration range of 0.1-1.0 $mg \cdot ml^{-1}$ for lentinan and laminarin used kinetic determinations. GNI had a *K*m of 9.0 $mg \cdot ml^{-1}$ with lentinan, whereas GNII had a *K*m value of 13.7 $mg \cdot ml^{-1}$ (Table V-2). The *K*m values for GNI and GNII with laminarin as substrate were 1.4 and 2.2 $mg \cdot ml^{-1}$, respectively. Thus, the *K*m values for GN I and II differed with lentinan and laminarin from *L. digitata* as substrates. The *K*m values for these β-glucanases with lentinan were approximately 6-fold higher than those with laminarin from *L. digitata*.

Table V-2. Kinetic properties of purified β-glucanases from *L. edodes*. Parameters were determined at 40°C in 30 mM sodium acetate buffer, pH 4.2, using 5.3 and 5.4 munits of GNI and II, respectively.

Substrate	β-glucanase	
	GN I	GN II
lentinan		
*K*m ($mg \cdot ml^{-1}$)	9.0	13.7
*K*m (μM) *	23.6	35.9
L. digitata laminarin *K*m ($mg \cdot ml^{-1}$)	1.4	2.2

* Based on a molecular mass for lentinan of 381 kDa by analysis of GPC

Table V-3. Relative rates of hydrolysis of β-glucan by purified β-glucanases from *L.edodes*. The relative rates of hydrolysis of the two glucanases against lentinan were arbitrarily set at 100% and correspond to 5.3 and 5.4 munits for GNI and II, respectively.

Substrate	Relative rate (%)	
	GN I	GN II
lentinan	100	100
L. digitata laminarin	827	678
CM curdlan	1.8	0.5
Pustulan	No activity	No activity
CM cellulose	No activity	No activity

Both glucanases hydrolyzed β-1, 3-glucans, lentinan from *L. edodes* and laminarin from *Laminaria digitata* (Table V-3). Laminarin from *L. digitata* was hydrolyzed at the highest rate by both glucanases (827

and 678 % compared with lentinan, respectively). CM-curdlan, however, was slightly degraded by both glucanases. In contrast, GNI and II did not attack against β-1, 6-glucan, pustulan, and β-1, 4-glucan, CM-cellulose. These results suggeste that the both GNI and II may hydrolyze specifically β-1, 3-linkages of glucan.

Maximum activities were observed at pH 4.2 for both β-glucanases (Figure V-4). More than 60% of maximum activity for both purified β-glucanases observed at range of pH 4.0 and 6.0.

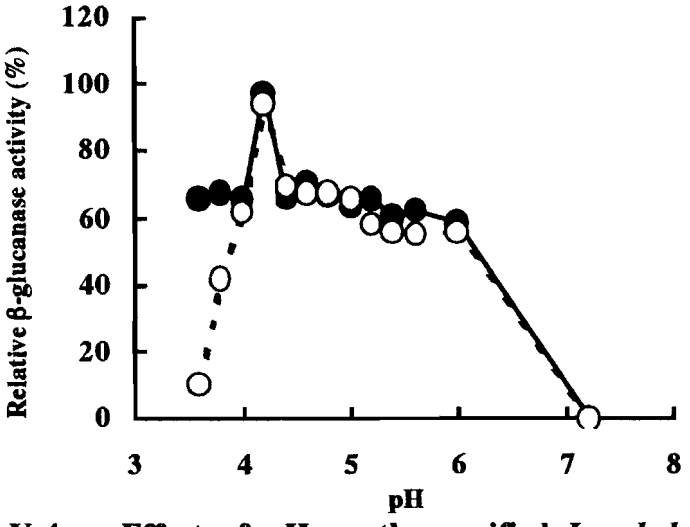


Figure V-4. Effect of pH on the purified *L. edodes* β–1, 3–glucanases. GNI activity; (●), GNII; (○). Maximum activities recorded for each enzyme were arbitrarily set at 100% and correspond to 5.3 and 5.4 munits of GNI and GNII, respectively.

Action patterns of purified β-1, 3-glucanases

In order to clarify the mode of action of GNI and II on lentinan, the identification of the enzymatic degradation products were attempted. Elution patterns of the degradation products on Sephacryl S-300 HR gel are shown in Figure V-5. On the elution patterns of the mixture incubated with GNI and II for 15 min (Figure V-5 A, C), two peaks were mainly detected, respectively, and the low molecular product was eluted at a range of less than 3 × 10^3 Da. After 48 hr of incubation, the peak corresponding to lentinan had almost

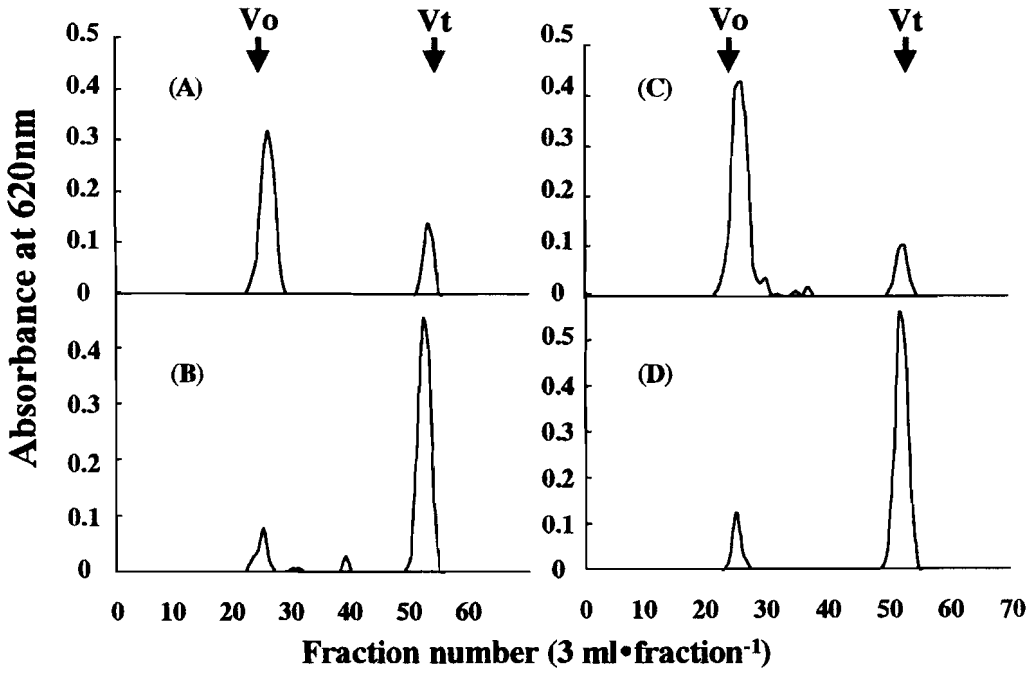


Figure V-5. Gel filtration pattern of the enzymatic digests of lentinan by *L. edodes* β-1, 3-glucanases. Authentic lentinan was incubated with GN I for 15 min; (A), 48 hr; (B) with GN II for 15 min; (C), 48 hr; (D). Amount of enzymes used were 5.3 and 5.4 munits for GN I and GN II, respectively.

disappeared, and the other peak was enhanced (Figure V-5 B, D). To identify these degradation products, the fractions of less than 3×10^3 Da of molecular masses, they were further subjected to HPLC analysis. A single peak on chromatogram was detected at retention time of about 6.4 min respectively, and they coincided with that of authentic glucose. These results suggest that the degradation product can be identical with glucose.

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

The synthesis pattern of GNI was examined in *L. edodes* fruiting body during storage, since the *K*m values of GNI and II indicated that the specificity of GNI against lentinan was higher than that of GNII. The western blotting analysis showed that GNI could not yet be synthesized in *L. edodes* fruiting body immediately after harvest (Figure

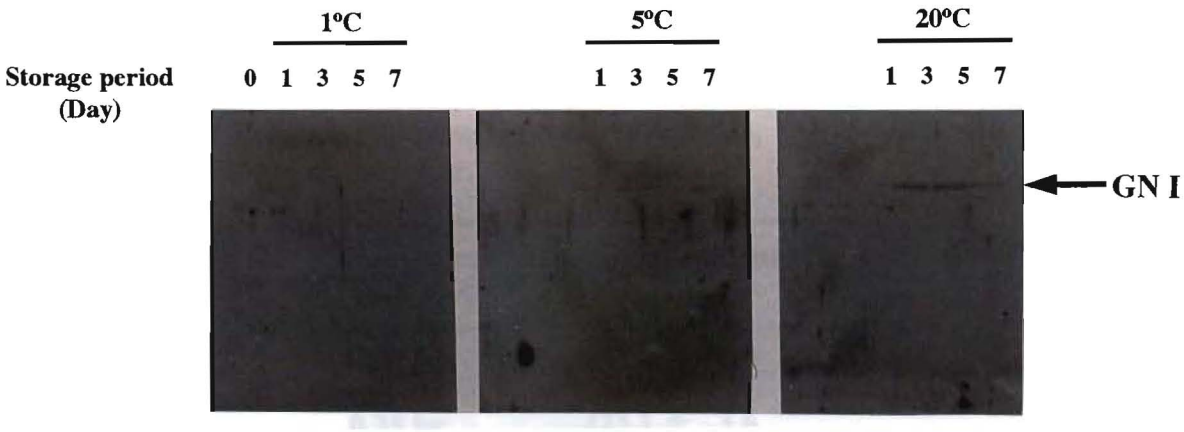


Figure V-6. The pattern of β-1, 3-glucanase synthesis in *L. edodes* fruiting bodies by western blot analysis. The fruiting bodies were stored for 7 days at 1, 5 and 20°C.

V-6). GNI has not been synthesized in the fruiting body during storage at 1°C for 7 days. It was synthesized at 5 days after storage at 5°C, and then its level increased. On the other hand, the GNI was synthesized during storage for one day at 20°C, and its level reached at highest level at 3 days and was kept during storage for 5 days. Thereafter, the level of GNI decreased gradually (Figure V-6). These results showed that β-1, 3-glucanase was synthesized *de novo* in *L. edodes* fruiting body under storage condition at 20°C.

The effect of enzymatically fragmented products from authentic lentinan on TNF-α and NO productions

When purified lentinan was incubated with GNI, its contents decreased (Table V-4). That is, lentinan content was approximately 500 $\mu g \cdot ml^{-1}$, and then it was rapidly degraded to 423.1 $\mu g \cdot ml^{-1}$ after incubation for 0.25 hr and decreased to 63.5 $\mu g \cdot ml^{-1}$ after incubation for 48 hr. Moreover, the immunomodulating effects of these incubation mixtures on TNF-α

Table V-4. Degradation of lentinan by the purified β-1, 3-glucanase. The authentic lentinan was incubated with 10 unit of GNI for 0.25, 0.5, 4, and 48 hr in 30 mM sodium acetate buffer, pH 4.2 at 40°C. Student's *t*-test: **P* < 0.025, ***P* < 0.002 and ****P* < 0.001 against 0 hr.

Incubation time (hr)	lentinan contents ($\mu g \cdot ml^{-1}$)
0 hr	537.8 ± 78.0
0.25 hr	423.1 ± 3.6
0.5 hr	344.9 ± 47.0*
4 hr	177.6 ± 34.7**
48 hr	63.5 ± 11.3***

and NO productions were measured. TNF-α productions from macrophages stimulated with lentinan were decreased with the decrease of lentinan content incubated with GNI (Figure V-7). TNF-α was released 248.9 ± 83.3 pg•ml^{-1} from macrophages stimulated with the mixture without incubation, which contained lentinan (approximately 500 μg•ml^{-1}). However, the amounts of TNF-α released from macrophages stimulated with the mixture after incubation for 0.25, 0.5, and 4 hr decreased to 214.6 ± 67.0, 148.4 ± 59.0 and 68.7 ± 20.8 pg•ml^{-1}, respectively. After 48 hr-incubation, TNF-α release decreased to 29.9 ± 6.9 pg•ml^{-1}.

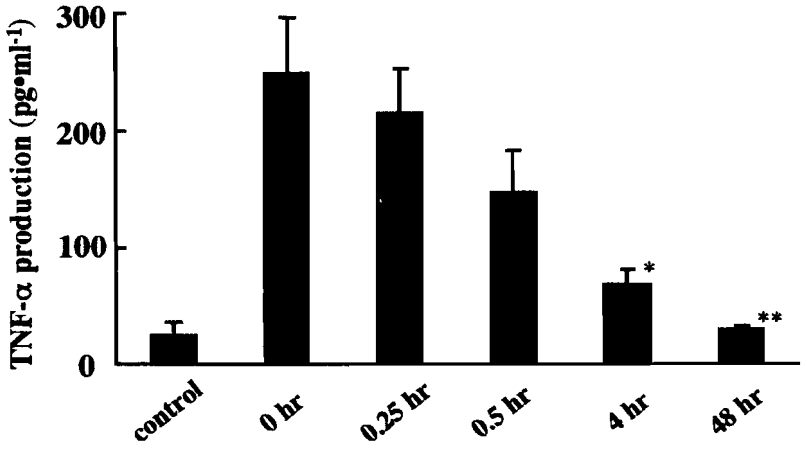


Figure V-7. Effect of lentinan incubated with β–1, 3-glucanase on TNF-α production from macrophages. Lentinan (500 μg•ml^{-1}) was incubated with GN I (10 units•ml^{-1}) in 30 mM NaOAc (pH4.2) at 40°C for 0.25, 0.5, 4, and 48 hr. All data points are mean + SD (n = 3). Student's *t*-test: *P < 0.025 and **P < 0.02 against 0 hr.

NO production from macrophages stimulated with lentinan was also decreased with lentinan degradation by GNI (Figure V-8). NO released 11.4 ± 2.8 μM from macrophages

stimulated with authentic lentinan (approximately 500 μg•ml^{-1}). NO production decreased with the elapse of incubation time of authentic lentinan and GNI, and the amounts of NO were 9.6 ± 2.8, 9.0 ± 2.8, and 5.0 ± 2.8 μM after 0.25 hr-, 0.5 hr-, and 4 hr-incubation, respectively. After 48hr-incubation of lentinan with the GNI, NO level was decreased to 2.0 ± 0.5 μM. These results suggested that lentinan was degraded enzymatically by β-1, 3-glucanase in *L. edodes*, and the decrease in lentinan content might be associated with a decrease of its immunomodulating effect on cytokines productions from macrophages.

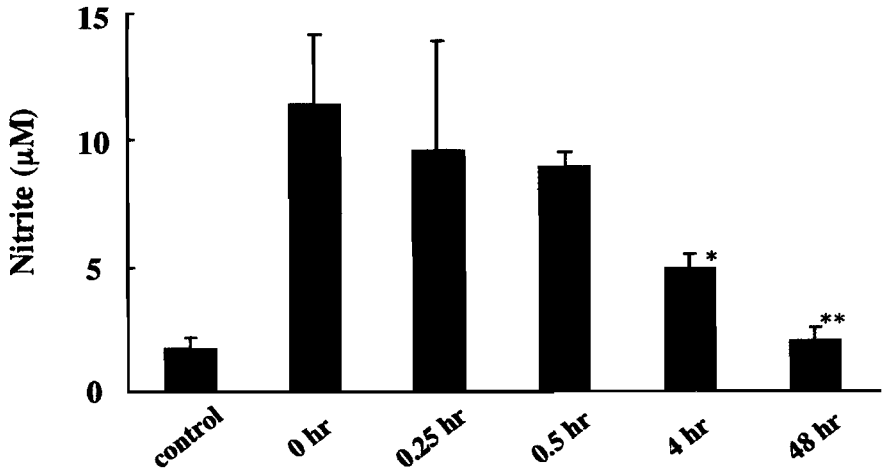


Figure V-8. Effect of lentinan incubated with β-1, 3-glucanase on NO production from macrophages. Lentinan (500 μg•ml^{-1}) was incubated with GN I (10 units•ml^{-1}) in 30 mM NaOAc (pH4.2) at 40°C for 0.25, 0.5, 4, and 48 hr. All data points are mean + SD (n = 3).
Student's *t*-test: **P* < 0.02, ***P* < 0.005 against 0 hr.

V-4 DISCUSSION

The author already described that an increase of β-glucanase activity in *L. edodes* fruiting bodies was related to the decrease of lentinan contents during storage of the mushroom at 20°C (in Chapter IV, Figures IV-3 and 7). In order to elucidate the function of β-glucanases in the degradation mechanism of lentinan in *L. edodes* during storage, these enzymes were isolated and purified from the fruiting body, and their some properties and synthesis pattern in *L. edodes* during storage were investigated. Two isozymes β–1, 3-glucanases, designated as GNI and GNII, were purified by $(NH_4)_2SO_4$ precipitation, anion-exchange and gel filtration chromatography (Scheme V-1 and Table V-1). The purity of the final enzyme preparations was confirmed by appearance of single protein bands on SDS-PAGE to be Mw 72,900 and 74,700, respectively, by SDS-PAGE. Comparison of these values with an apparent Mw 70,000 obtained for the two isoenzymes by gel-filtration chromatography (Figure V-3) indicates that they are monomeric proteins. Purification factors for the each enzyme are 93.8 and 47.6 for GNI and II, respectively (Table V-1).

Although a few fungal β-glucanases have been as extensively characterized, a comparison of their physico-chemical properties with those found for the *L. edodes* GNs reveals some similarities. The molecular weight and optimatic pH of each purified *L. edodes* GN were similar and fell well within the range of values previously reported for other β-glucanases (Bielrcki and Galas, 1991, Stone and Clarke, 1992 and Pitson *et al.*, 1993). For example, it has been reported that the molecular weight of fungal β-glucanases were commonly in the range of 20-80 kDa (Pitson *et al.*, 1993, 1995). Similarly, the majority of

fungal β-glucanases have optimal activity between pH 4.0-6.0 (Bielrcki and Galas, 1991 and Pitson *et al.*, 1993 and 1995).

The substrate specificities and action patterns of the *L. edodes* GNs have been compared using the linear (laminarin and pustulan), the substituted (CM-curdlan and CM-cellulose) and the side-branched (lentinan) β-glucans. A single peak on the chromatgram was detected at a retention time corresponding to that of authentic glucose, as determined by HPLC analysis of degradation products liberated enzymatically from lentinan (data not shown). 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 the degradation mixture digested for 15 min and 48 hr (Figure V-5). These results suggest that both purified GNs act in an exo-hydrolytic manner, removing single glucose residues from non-reducing end of lentinan as determined by product analysis. The preferred substrate for the two isoenzymes is *L. digitata* laminarin (Table V-3), an essentially linear, soluble, β-1, 3-D-glucan with a low degree of glycosyl substitution at *O*-6. Their activities of the *L. edodes* GNs for laminarin were approximately 7-8 times higher than those for lentinan (Table V-3). In contrast, the GNs hydrolyzed CM-curdlan at significant low rate. These results suggested that β-1, 6-glucose branches and CM substituents in β-1, 3-glucan might interfere to produce enzyme-substrate interactions. The *L. edodes* GNs had no activity on pustulan, β-1, 6-glucan, and CM-cellulose, substituted β-1, 4-glucan. These results confirmed that the purified β-glucanase isoenzymes were classified into *exo* type β-1, 3-glucanase. Most

fungi appear to secrete both *exo*- and *endo*-glucanases (Pitson *et al.*, 1993), and, these may act synergistically for efficient degradation of simple and complex β-D-glucans (Copa-Patinõ *et al.*, 1990). However, *L. edodes* contained only *exo* type β-1, 3-glucanases, as well as in *A. persicinum* glucanases (Pitson *et al.*, 1995). In addition to, both GNs had *K*m values with lentinan about six times higher than those with laminarin (Table V-2). These are probably due to a reflection of the structure of lentinan possessing β-1, 6-glucose branches with β-1, 3-glucan backbone. The *K*m values for GN I with lentinan was lower than that for GN II (Table V-2), suggesting that the GN I seemed to hydrolyze more specifically lentinan than GN II.

The author already reported that an increase of β-glucanase activity in *L. edodes* fruiting bodies might be related to lentinan decrease during storage in Chapter IV. As shown in Figure V-6, β-1, 3-glucanase was synthesized and accumulated more significantly in *L. edodes* fruiting body during storage at 20°C. This result showed that an increase of β-glucanase activity was certainly associated with an increase of β-1, 3-glucanase content during storage of *L. edodes* at 20°C. Therefore, it is suggested that β–1, 3-glucanase was synthesized *de novo* in *L. edodes* and lentinan was degraded during storage at high temperature such as 20°C.

As described previously in Chapter IV, the author elucidated that the immunomodulating activities of *L. edodes* decreased during storage at 20°C, and the decrease would be related to the decrease of lentinan content. In this chapter, the immunomodulating effects of the enzymatically degraded products from authentic lentinan

on cytokines productions diminished (Table V-4, Figures V-7 and 8). These results showed that degradation of lentinan by β-1, 3-glucanase resulted in a decrease in the immunomodulating activity of *L. edodes* during storage at 20°C. Sasaki *et al* (1976) reported that "small lentinan", which was hydrolyzed with formic acid to a molecular weight of $1\times 10^4 \sim 6 \times 10^3$ Da, was possessed of antitumor activity, but lentinan of molecular weight of less than 6×10^3 Da did not show antitumor activity. However, the decrease of immunomodulating activity of *L. edodes* seemed to occur as a result of the decrease in lentinan content with the degradation by *exo* β-1, 3-glucanase during storage of *L. edodes*.

On the basis of these observations, the author finally concludes that the β-1, 3-glucanase is synthesized *de novo* in *L. edodes* fruiting body during storage at high temperature such as 20°C, and this enzyme attacks lentinan by the manner of *exo* type to produce only glucose (Figure V-9). As the result, the molecular size of lentinan changes to smaller one, and its content decreases. This will be a reason why the immunomodulating activities of *L. edodes* decrease during storage.

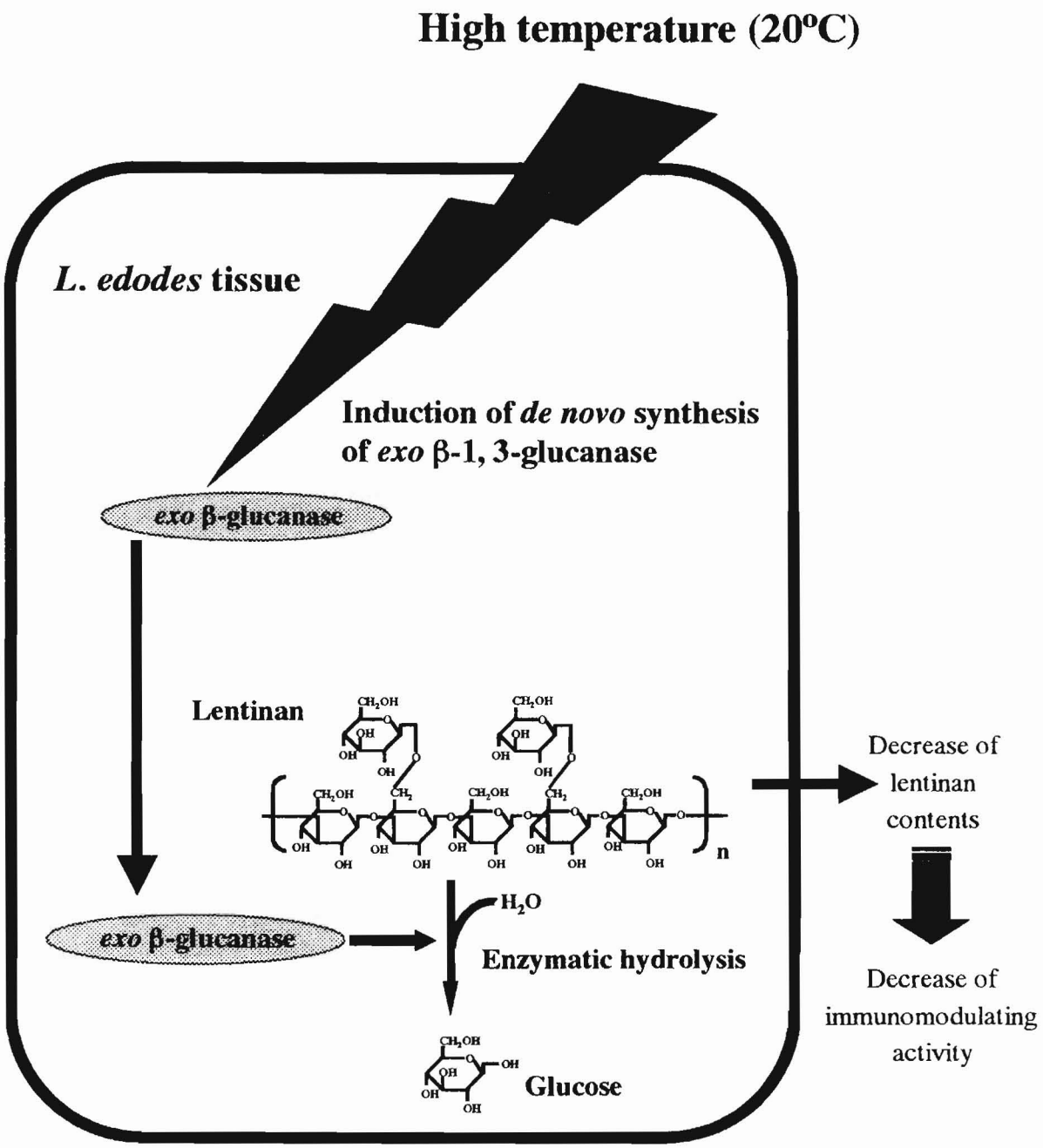


Figure V-9. The hypothesis of lentinan degradation in *L. edodes* during storage at high temperature.

VI CONCLUSION

Many investigators have found that the polysaccharides in several mushrooms possessed strong antitumor activity, and these polysaccharides mainly were β-1, 3- or β-1, 6-glucan. As it is desired that the mushroom is utilized as functional food more efficiently, it is necessary to clarify the behavior of the antitumor polysaccharide in the mushroom fruiting body during growth and storage, and to elucidate the difference of its content among strains of the mushroom.

However, it has been difficult that a polysaccharide was determined simply and reproducibly, since the purification and identification of polysaccharides from the fruiting bodies of mushrooms were very tedious and difficult. First of all, in Chapter II, the author attempted to establish the method for quantification of lentinan in *L. edodes* and GGF in *G. frondosa*, respectively, with high specificity and reproducibility by enzyme linked immunosorbent assay (ELISA) using anti-lentinan and anti-GGF antibodies. These antibodies were raised in rabbits by subcutaneous immunization. The anti-lentinan antibody showed high specificity for only lentinan, but they did not react with laminarin, the essentially linear β-1, 3-glucan that was recognized as the backbone of lentinan. This result suggested that an anti-lentinan antibody recognized the site of β-1, 6-branches in β-1, 3-glucan backbone of lentinan as an antigen epitope. And, the anti-GGF antibody recognized GGF significantly, and it reacted with lentinan somewhat. However, it did not react with pustulan, the linear β-1, 6-glucan, suggesting that this antibody recognized the site

of β-1, 3-branches in the β-1, 6-glucan backbone of GGF.

Although at first the author attempted to measure lentinan in *L. edodes* extract by ELISA direct assay, it could not be measured sufficiently. This reason was considered that the contaminant in the extract interfered with the specific reaction of the antibody with lentinan. Therefore, the author attempted to make use of inhibition assay of ELISA for quantification of lentinan and GGF. And, the author found these polysaccharides in mushrooms could be quantified with dose dependency by this method. These results suggest that the use of inhibition assay of ELISA as specific immunoassay, which is less complicate method than a sandwich and the other methods in ELISA, can be advantageous to the easy and simple quantification of an antitumor polysaccharide. Hence, it was elucidated that the inhibition assay of ELISA were applicable to clarifications of following matters in Chapter III and IV;

(1) The determination of the contents of lentinan-like and GGF-like polysaccharides in several mushrooms. (Chapter III)

(2) The changes in lentinan and GGF contents in *L. edodes* and *G. frondosa*, respectively, during growth of the mushroom fruiting bodies. (Chapter III)

(3) The changes in lentinan and GGF contents in *L. edodes* and *G. frondosa*, respectively, during storage of the mushrooms. (Chapter IV)

Moreover, the following problem was clarified by the immunohistochemical analysis using the prepared antibody;

(4) The localization of lentinan in *L. edodes* tissue. (Chapter III)

In Chapter III, it was elucidated four of eleven mushrooms contained lentinan-like polysaccharide more than the others. The content of lentinan in *L. edodes* was at the highest level (3.4 $mg \bullet g^{-1}$ f.w.), and its contents were high with successive in *Flammulina velutipes* "Enokitake", *Lactarius hatsudake* "Hatsudake" and *Sarcodon aspratus* "Koutake", containing 2.5, 2.3 and 2.2 $mg \bullet g^{-1}$ f.w., respectively. And, the contents of GGF-like polysaccharide in four mushrooms were higher than in *G. fromdosa* (2.4 $mg \bullet g^{-1}$ f.w.), that is, 7.3 $mg \bullet g^{-1}$ f.w. in *Panellus serotinus* "Mukitake", 6.6 $mg \bullet g^{-1}$ f.w. in *F. velutipes*, 4.1 $mg \bullet g^{-1}$ f.w. in *L. hatsudake* and 3.0 $mg \bullet g^{-1}$ f.w. in *L. edodes*. However, both lentinan-like and GGF-like polysaccharide was little contained in *Agaricus brazei* "Himematsutake", *Agaricus bisporus* "Tsukuritake (so called Mushroom)" and *Ramaria botrytis* "Houkitake". *R. botrytis*, *G. fromdosa*, *S. aspratus* and *Meripilus giganteus* "Tonbimaitake" belong to the family of Aphyllophorales, and the others to Agaricales. These results suggested that there was no relationship between the contents of antitumor polysaccharides and the family of the mushrooms.

It was also shown that the content of lentinan during growth increased to peaks (18.0 mg in one fruiting body) at the growth stage II of *L. edodes*. However, its content decreased to 15.9 mg at the growth stage III. Although GGF content increased to 4.8 mg in one fruiting body at the growth stage III, its content decreased to 2.7 mg at the stage IV during growth of *G. frondosa*. Moreover, in Chapter III, it was clarified that lentinan localized mainly in the site of cap of *L. edodes*, but it did not appreciably accumulate in the site of stipe.

In Chapter IV, it was elucidated that the content of lentinan was maintained during storage of *L. edodes* at low temperatures (1 and 5°C), but it decreased dramatically at high temperature (20°C). The changes in GGF content during storage of *G. frondosa* showed the same tendency as the changes in lentinan contents during storage. These results suggested that low temperature storage was effective to maintain antitumor polysaccharides contents in mushrooms.

These results described above demonstrated that the inhibition assay of ELISA established in this study could be sufficiently applied to the quantification of an antitumor polysaccharide in a mushroom. And also, it was demonstrated that the immunohistochemical analysis could detect *in situ* the targeting polysaccharide in the mushroom tissue.

In Chapter IV, moreover, it was shown that an increase in β-glucanase activity seemed to be related to a decrease in lentinan content during storage of *L. edodes.* Therefore, in order to elucidate the mechanism of lentinan degradation, the author attempted to isolate and purify this enzyme, and investigated some properties of this enzyme and its synthesis pattern during storage of *L. edodes.* In Chapter V, two β-1, 3-glucanase isozymes (designed as GNI and GNII, respectively) were isolated and purified from *L. edodes* fruiting bodies, and they both were found to be monomeric proteins and 70kDa of molecular weights by gel filtration and SDS-PAGE. It was found that both enzymes attacked β-glucan in *exo* manner, since only glucose was identified as enzymatic product released from lentinan. *K*m values for GNI and GNII against lentinan were 9.0 and 13.7

$mg \cdot ml^{-1}$ respectively, suggesting that GNI hydrolyzed lentinan more specifically than GNII. GNI, moreover, was found to be synthesized *de novo* in fruiting body during storage of *L. edodes* at 20°C by western blotting analysis, and its pattern coincided with the change pattern of β-glucanase activity during storage. These results demonstrated that an increase in β-1, 3-glucanase activity was closely associated with a decrease in lentinan content during storage of *L. edodes*.

Lentinan is recognized as immunomodulator, since its antitumor activity considers to be exerted through activation of immunocompetent cells such as macrophages in immune system. One of its immunomodulating activities is shown as the productions of cytokines, such as TNF-α and NO etc., from immunocompetent cells. In Chapter IV, it was found that the immunomodulating effects of *L. edodes* on TNF-α and NO productions from macrophages stimulated with stored *L. edodes* extracts diminished with the decrease in lentinan content during storage. In addition, in Chapter V, the authentic lentinan was degraded by the β-1, 3-glucanase from *L. edodes*, and its immunomodulating activity in cytokines productions diminished with lentinan degradation. On the basis of these results, the author concluded that *exo* β-1, 3-glucanase was synthesized *de novo* in *L. edodes* during storage at high temperature such as 20°C, and the increase in this enzyme activity was related closely to the decrease in lentinan contents and the immunomodulating activity of *L. edodes* during storage.

REFERENCES

Ábel, G., Szöllősi, J., Chihara, G., and Fachet, J. (1989) Effect of lentinan and mannan on phagocytosis of fluorescent latex microbeads by mouse peritoneal macrophages: a flow cytometric study. *Int. J. Immunopharmacol.* **11**, 615-621.

Adachi, Y., Ohno, N., Ohsawa, M., Sato, K., Oikawa, S., and Yadomae, T. (1989) Physicochemical properties and antitumor activities of chemically induced derivatives of antitumor glucan "Grifolan LE" from *Grifola frondosa. Chem. Pharm. Bull.***37**, 1838-1843.

Adachi, Y., Ohno, N., and Yadomae, T. (1994) Preparation and antigen specificity of an anti-(1-3)-β-D-glucan antibody. *Biol. Pharm. Bull.* **17**, 1508-1512.

Adachi, Y., Okazaki, M., Ohno, N., and Yadomae, T. (1994) Enhancement of Cytokine production by macrophages stimulated with (1-3)-β-D-glucan, grifolan (GRN), isolated from *Grifola frondosa. Biol. Pharm. Bull.* **17**, 1554-1560.

Aketagawa, J., Tanaka, S., Tamura, H., Shibata, Y., and Saito, H. (1993) Activation of limulus coagulation factor G by several (1-3)-β-D-glucans : comparison of the potency of glucans with identical degree of polymerization but different conformation. *J. Biochem.* **113**, 683-686.

Asai, K., Kato, H., Kimura, S., Mukai, S., Kawahito, Y., Sano, H., Kondo, M., Akaogi, K., and Hirose, K. (1996) Induction of gene expression for nitric oxide synthase by immunomodulating drugs in the RAW264.7 murine macrophage cell line. *Cancer Immunol. Immunother.* **42**, 275-279.

Azuma, I. (1987) Development of immunostimulants in Japan. In *Immunostimulants Now and Tomorrow*, Azuma I. and Jolles G. (eds), pp. 41-56, Japan Scientific Societies Press, Tokyo.

Bielrcki, S., and Galas, E. (1991) Microbial β-glucanases different from cellulases. *Crit. Rev. Biotechnol.* **10**, 275-304.

Borchers, A. T., Stern, J. S., Hackman, R. M., Keen, C. L., and Gershwin, M. E. (1999) Mushrooms, tumors, and immunity. *Proc. Soc. Exptl. Biol. Med.* **221**, 281-291.

Bradford, M. M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.

Carswell, E. A., Old, L. J., Kassel, R. L., Green, S., Fiore, N., and Williamson, B. (1975) An endotoxin-induced serum factor that causes necrosis of tumors. *Proc. Natl. Acad. Sci. U.S.A.* **72**, 3666-3670.

Chihara, G., Maeda, Y., Hamuro, J., Sasaki, T., and Fukuoka, F. (1969) Inhibition of mouse Sarcoma 180 by polysaccharides from *Lentinus edodes* (Berk.) Sing. *Nature* **222**, 687-688.

Chihara, G., Hamuro, J., Maeda, Y. Y., Arai, Y., and Fukuoka, F. (1970) Fractionation and purification of the polysaccharides with marked antitumor activity, Especially lentinan, from *Lentinus edodes* (Berk.) Sing. (an edible mushroom). *Cancer Res.* **30**, 2776-2781.

Chihara, G., Suga, T., and Hamuro, J. (1987) Antitumor and metastasis inhibitor, activities of lentinan as an immunomodulator. *Cancer Detect. Pre. Suppl.* **1**, 423.

Copa-Patinõ, J. L., Rodriquez, J., Reyes, F., and Perez-Leblic, M. I. (1990) Effect of β-glucanases on *Penicillium oxalicum* cell wall fractions. *FEMS Microbiol lett.*, **70**, 233-240.

Dennert, G., and Tucker, D. (1973) Antitumor polysaccharide lentinan – a T-cell adjuvant. *J. Nati. Cancer Inst.* **51**, 1727-1729.

Di Luzio, N. R. (1985) Update on the immunomodulating activities of glucans. *Springer Sem. Immunopath.* **8**, 387-400.

Douwes, J., Doekes, G., Montijn, R., Heederik, D., and Brunekreef, B. (1996) Measurement of β(1-3)-glucans in occupational and home environments with an inhibition enzyme immunoassay. *Appl. Environ. Microbiol.* **62**, 3176-3182.

Dresser, D. W., and Phillips, J. M. (1974) The orientation of the adjuvant activities of Salmonella typhosa liposaccharide and lentinan. *Immunology* **27**, 895-902.

Freunhauf, P. J., Bonnard, D. G., and Heberman, B. R. (1982) The effect of lentinan on production of interleukin-1 by human monocytes. *Immunopharmacol.* **5**, 65-74.

Gergely, P., Vallent, K., Bodo, I., Feher, J., and Kaneko, Y. (1988) Effects of lentinan on cytotoxic functions of human lymphocytes. *Immunopharmacol. Immunotoxicol.* **10**, 157-163.

Gong, Y., Abe, K., and Chachin, K. (1993) Changes in phenol oxidases activity with special reference to browning of shiitake (*Lentinus edodes* Sing.) mushroom during storage. *J. Jpn. Soci. Cold Preserv. Food* **19**, 74-78.

Green, C. L., Wagner, A. D., Glogowski, J. Skipper, L. P., Wishnok, S. J., and

Carswell, E. A., Old, L. J., Kassel, R. L., Green, S., Fiore, N., and Williamson, B. (1975) An endotoxin-induced serum factor that causes necrosis of tumors. *Proc. Natl. Acad. Sci. U.S.A.* **72**, 3666-3670.

Chihara, G., Maeda, Y., Hamuro, J., Sasaki, T., and Fukuoka, F. (1969) Inhibition of mouse Sarcoma 180 by polysaccharides from *Lentinus edodes* (Berk.) Sing. *Nature* **222**, 687-688.

Chihara, G., Hamuro, J., Maeda, Y. Y., Arai, Y., and Fukuoka, F. (1970) Fractionation and purification of the polysaccharides with marked antitumor activity, Especially lentinan, from *Lentinus edodes* (Berk.) Sing. (an edible mushroom). *Cancer Res.* **30**, 2776-2781.

Chihara, G., Suga, T., and Hamuro, J. (1987) Antitumor and metastasis inhibitor, activities of lentinan as an immunomodulator. *Cancer Detect. Pre. Suppl.* **1**, 423.

Copa-Patinõ, J. L., Rodriquez, J., Reyes, F., and Perez-Lœblic, M. I. (1990) Effect of β-glucanases on *Penicillium oxalicum* cell wall fractions. *FEMS Microbiol lett.*, **70**, 233-240.

Dennert, G., and Tucker, D. (1973) Antitumor polysaccharide lentinan – a T-cell adjuvant. *J. Nati. Cancer Inst.* **51**, 1727-1729.

Di Luzio, N. R. (1985) Update on the immunomodulating activities of glucans. *Springer Sem. Immunopath.* **8**, 387-400.

Douwes, J., Doekes, G., Montijn, R., Heederik, D., and Brunekreef, B. (1996) Measurement of β(1-3)-glucans in occupational and home environments with an inhibition enzyme immunoassay. *Appl. Environ. Microbiol.* **62**, 3176-3182.

Dresser, D. W., and Phillips, J. M. (1974) The orientation of the adjuvant activities of Salmonella typhosa liposaccharide and lentinan. *Immunology* **27**, 895-902.

Freunhauf, P. J., Bonnard, D. G., and Heberman, B. R. (1982) The effect of lentinan on production of interleukin-1 by human monocytes. *Immunopharmacol.* **5**, 65-74.

Gergely, P., Vallent, K., Bodo, I., Feher, J., and Kaneko, Y. (1988) Effects of lentinan on cytotoxic functions of human lymphocytes. *Immunopharmacol. Immunotoxicol.* **10**, 157-163.

Gong, Y., Abe, K., and Chachin, K. (1993) Changes in phenol oxidases activity with special reference to browning of shiitake (*Lentinus edodes* Sing.) mushroom during storage. *J. Jpn. Soci. Cold Preserv. Food* **19**, 74-78.

Green, C. L., Wagner, A. D., Glogowski, J. Skipper, L. P., Wishnok, S. J., and

Tannebaum, R. S. (1982) Analysis of nitrate, nitrit and [^{15}N] nitrate in biological fluids. *Anal. Biochem.* **126**, 131-138.

Haba, S., Hamaoka, T., Takatsu, K., and Kitagawa, M. (1976) Selective suppression of T-cell activity in tumor-bearing mice and its improvement by lentinan. *Int. J. Cancer* **18**, 93-104.

Hamuro, J., and Chihara, G. (1984) Lentinan, a T-cell oriented immunopotentiator: its experimental and clinical application, and possible mechanism of immune modulation. In *Immune Modulation Agents*, R. L. Fenichel and M. A. Chirigos (eds.), pp. 409-436, New York, Marcel Dekker Inc.

Hamuro, J., Röllinghoff, M., and Wagner, H. (1978) β(1-3) glucan-mediated augmentation of alloreactive murine cytotoxic T-lymphocytes *in vivo*. Cancer Res. **38**, 3080-3085.

Hamuro, J., Wagner, H., and Röllinghoff, M. (1978) β(1-3)glucan as a probe for T-cell specific immune adjuvant: enhanced *in vitro* generation of cytotoxic T-lymphocytes. *Cell Immunol.* **38**, 328-335.

Herlyn, D., Kaneko, Y., Rewe, J., Aoki, T., and Koprouski, H. (1983) Monoclonal antibody-dependent murine macrophage-mediated cytotoxicity against human tumors is stimulated by lentinan. *Gann* **76**, 37-42.

Hirata, A., Itoh, W., Tabata, K., Kojima, T., Itoyama, S., and Sugawara, I. (1993) Preparation and characterization of murine anti-schizophyllan monoclonal antibody, SPG-HS. *Biosci. Biotech. Biochem.* **57**, 125-126.

Hoffman, O. A., Standing, J. E., and Limper, A. H. (1993) *Pneumocystis carinii* stimulates tumor necrosis factor-α release from alveolar macrophages through a β-glucan-mediated mechanism. *J. Immunol.* **150**, 3932-3940.

Ikekawa, T., Nakanishi, M., Uehara, N., Chihara, G., and Fukuoka, F. (1968) Antitumor action of some Basidiomycetes, especially *Phllinus inteus. Jpn. J. Cancer Res. (Gann)* **59**, 155-157.

Ikekawa, T., Uehara, N., Maeda, Y., Nakanishi, M., and Fukuoka, F. (1969) Antitumor activity of aqueous extracts of edible mushrooms. *Cancer Res.* **29**, 734-735.

Ikekawa, T., Ikeda, Y., Yoshioka, Y., Nakanishi, K., Yokoyama, E., and Yamazaki, E. (1982) Study on antitumor polysaccharides of *Flammulina velutipes* (Curt. ex Fr.) Sing. II. The stracture of EA 3 and further purification of EA 5. *J. Pharmacobio-Dyn.* **5**, 576-581.

Irinoda, K., Masihi, K. N., Chihara, G., Kaneko, Y., and Katori, T. (1992) Stimulation of microbicidal host deffence mechanisms against aerosol influenza virus infection by lentinan. *Int. J. Immunopharmac.* **14**, 971-977.

Ito, H., Shimura, K., Itoh, H., and Kawade, M. (1997) Antitumor effects of a new polysaccharide-protein complex (ATOM) prepared from *Agaricus brazei* (Iwade strain 101) "Himematsutake" and its mechanism in tumor-bearing mice. *Anticancer. Res.* **17**, 277-284.

Kawagishi, H., Inagaki, R., Kanno, T., and Mizuno, T. (1986) Fractionation and antitumor activity of the water-insoluble of *Agaricus blazei* fruiting bodies. *Carbohydr. Res.* **186**, 267-273.

Kawai, M. (1970) Mycolytic enzymes produced by some stains of Coprinus. 2. Characteristics of crude enzymes produced by *Coprinus macrorhizus* f. *microsporus* and *Coprinus radians. Hakko kogaku zasshi*, **48**, 397-404.

Kawai, M., and Ishida, K. (1970) Mycolytic enzymes produced by some stains of Coprinus. 3. Treatment of bacteria and yeast cells by crude enzymes produced by *Coprinus macrorhizus* f. *microsporus* Hongo and *Coprinus radians. Hakko kogaku zasshi*, **48**, 405-410.

Kerékgyártó, C., Virág, L., Tankó, L., and Chihara, G. (1996) Strain differences in the cytoto
xic and TNF production of murine macrophages stimulated by lentinan. *Int. J. Immunopharmac.* **18**, 347-353.

Kiho, T., Katsuragi, M., Nagai, K., Ukai, S., and Haga, M. (1992) Structure and antitumor activity of a branched (1-3)-β-D-glucan from the alkaline extract of *Amanita muscaria. Carbohyd. Res.* **224**, 237-243.

Kikuchi, S., Ohinata, A., Tsumuraya, Y., Hashimoto, Y., Kaneko, Y., and Matsushima, H. (1993) Production and characterization of antibodies to the β-(1-6)-galactotetraosyl group and their interaction with arabinogalactan-proteins. *Planta* **190**, 525-535.

Kitamura, S., Hori, T., Kurita, K., Takeo, K., Hara, C., Itoh, W., Tabata, K., Elgsaeter, A., and Stokke, B. (1994) An antitumor, branched (1-3)-β-D-glucan from a water extract of fruiting bodies of *Cryptoporus volvatus. Carbohydr. Res.* **263**, 111-121.

Komatsu, N., Okubo, S., Kikumoto, S., Kimura, K., and Saito, G. (1969) Host-mediated antitumor action of schizophyllan, a glucan produced by *Schizophyllum commune. Gann* **60**, 137-144.

Ladányi, A., Tímár, J., and Lapis, K. (1993) Effect of lentinan on macrophage cytotoxicity

against metastatic tumor cells. *Cancer Immunol. Immunother.* **36**, 123-126.

Laemmli, U. K. (1970) Cleavege of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Liu, F., Ooi, V. E. C., Liu, W. K., and Chang, S. T. (1996) Immunomodulation and antitumor activity of polysaccharide-protein complex from the culture filtrates of a local edible mushroom, *Tricholoma lobayense. Gen. Pharmac.* **27**, 621-624.

Leung, M. Y. K., Fung, K. P., and Choy, Y, M. (1997) The isolation and characterization of an immunomodulating and anti-tumor polysaccharide preparation from *Flammulina velutipes. Immunoparmacolgy* **35**, 255-263.

Lowenstein, C. J., and Snyder, S. H. (1992) Nitric oxide, a novel biologic messenger. *Cell* **70**, 705-707.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. L. (1951) Protein measurement with Folin phenol reagent. *J. Biol. Chem.* **193**, 265-275.

Maeda, Y. Y., and Chihara, G. (1971) Lentinan, a new immunoaccelerator of cell-mediated responses. *Nature* **229**, 634-635.

Maeda, Y. Y., and Chihara, G. (1973) The effects of neonatal thymectomy on the antitumor activity of lentinan, carboxymethyl pachymaran and Zymosan and their effects on various immune responses. *Int. J. Cancer* **11**, 153-161.

Maeda, Y. Y., Chihara, G., and Ishimura, K. (1974) Unique increase of serum proteins and action of antitumor polysaccharides. *Nature* **252**, 250-252.

Maeda, Y. Y., Hamuro, J., and Chihara, G. (1971) The mechanisms of action of antitumor polysaccharides: I. The effects of antilymphocyte serum on the antitumor activity of lentinan. *Int. J. Cancer* **8**, 41-46.

Maruyama, H., Yamazaki, K., Murofushi, S., Konda,C., and Ikekawa, T. (1989) Antitumor activity of *Sarcodon aspratus* (Berk.) S. Ito and *Ganoderma lucidum* (Fr.) Karst. *J. Pharmacobio-Dyn.* **12**, 118-123.

Meikle, P. J., Bonig, L., Hoogenraad, N. J., Clarke, A. E., and Stone, B. A. (1991) The location of (1-3)-β-glucans in the walls of pollen tubes of *Nicotiana alata* using a (1-3)-β-glucan-specific monoclonal antibody. *Planta* **185**, 1-8.

Mikami, T., Nagase, T., Matsumoto, T., Suzuki, S., and Suzuki, M. (1982) Gelation of

Limulus amoebocyte lysate by simple polysaccharides. Microbiol. Immunol. **26**, 403-409.

Minamide, T., Habu, T., and Ogata, K. (1980a) Effect of storage temperature on keeping freshness of mushrooms after harvest. *J. Jpn. Soci. Food Sci. Tech.* **27**, 281-287.

Minamide, T., Tsuruta, M., and Ogata, K. (1980b) Studies on keeping freshness of Shii-take mushroom (*Lentinus edodes* (Bark) Sing.) after harvest. *J. Jpn. Soci. Food Sci. Tech.*, **27**, 498-504.

Minamide, T., Nishikawa, T., and Ogata, K. (1980c) Influences of CO_2 and O_2 on the keeping freshness of Shii-take mushroom (*Lentinus edodes* (Bark) Sing.) after harvest. *J. Jpn. Soci. Food Sci. Tech.* **27**, 505-510.

Misaki, A., Kakuta, M., Sasaki, T., Tanaka, M., and Miyaji, H. (1981) Study on interrelation of structure and antitumor effects of polysaccharides: antitumor action of periodate-modified, branched (1-3)-β-D-glucan of *Auricularia auricula-judae*, and other polysaccharides containing (1-3)-glycosidic linkages. *Carbohydr. Res.* **9**, 115-129.

Mizuno, T., Kato, N., Tostuka, A., Takenaka, K., Shinkai, K., and Shimizu, M. (1984) Fractionation, structural features and antitumor activity of water-soluble polysaccharide from "Reishi," the fruit body of *Ganoderma lucidum*. *Nippon Nogeikagaku Kaishi* **58**, 871-880.

Mizuno, T., Inagaki, R., Kanao, T., Hagiwara, T., Nakamura, T., Ito, H., Shimura, K., Sumiya, T., and Asakura, A. (1990a) Antitumor activity and some properties of water-soluble polysaccharides from "Himematsutake," the fruiting body of *Agaricus blazei* Murill. *Agric. Biol. Chem.* **54**, 2889-2896.

Mizuno, T., Inagaki, R., Kanao, T., Hagiwara, T., Nakamura, T., Ito, H., Shimura, K., Sumiya, T., and Asakura, A. (1990b) Antitumor activity and some properties of water-insoluble hetero-glycans from "Himematsutake," the fruiting body of *Agaricus blazei* Murill. *Agric. Biol. Chem.* **54**, 2897-2905.

Mizuno, T., Ando, M., Sugie, R., Ito, H., Shimura, K., Sumiya, T., and Matsuura, A. (1992) Antitumor activity of some polysaccharides isolated from an edible mushroom, *Ningyotake*, the fruiting body and the cultured mycelium of *Polyporous confluens*. *Biosci. Biotech. Biochem.* **56**, 34-41.

Mizuno, T., Yeohlui, P., Kinoshita, T., Zhuang, C., Ito, H., and Mayuzumi, Y. (1995) Antitumor activity and chemical modification of polysaccharides from *Niohshimeji* mushroom, *Tricholma giganteum*. *Biosci. Biotech. Biochem.* **60**, 30-33.

Mizuno, M., Morimoto, M., Minato, K. and Tsuchida, H. (1998) Polysaccharides from

Agaricus blazei stimulate lymphocyte T-cell subsets in mice. *Biosci. Biotechnol. Biochem.*, **62**, 434-437.

Mizuno, M., Shiomi, Y., Minato, K., Kawakami, S., Ashida, H. and Tsuchida, H. (1999) Fucogalactan isolated from *Sarcodon aspratus* elicits release of tumor necrosis factor-α and nitric oxide from murine macrophages. *Immunopharmcology*, **in press.**

Nagi, N., Ohno, N., Adachi, Y., Aketagawa, J., Tamura, H., Shibata, Y., Tanaka, S., and Yadomae, T. (1994) Application of *Limulus* test (G pathway) for the detection of different conformers of (1-3)-β-D-glucans. *Biol. Pharm. Bull.* **16**, 822-828.

Nanba, H., Hamaguchi, A., and Kuroda, H. (1987a) The chemical structure of an antitumor polysaccharide in fruit bodies of *Grifola frondosa* (Maitake). *Chem. Phrm. Bull.* **35**, 1162-1168.

Nanba, H., Mori, K., Toyomasu, T., and Kuroda, H. (1987b) Antitumor action of shiitake (*Lentinus edodes*) fruit bodies orally administered to mice. *Chem. Pharm. Bull.* **35**, 2453-2458.

Nanba, H., and Kuroda, H. (1987c) Antitumor mechanisms of orally administered shiitake fruit bodies. *Chem. Pharm. Bull.* **35**, 2459-2464.

Nanba, H. and Kuroda, H. (1988) Potentiation of host-mediated antitumor activity by orally administered mushroom (*Agaricus bispora*) fruit bodies. *Chem. Pharm. Bull.*, **36**, 1437-1444.

Nelson, N. (1944) A photometric adaptation of Somogyi method for the determination of glucose. *J. Biol. Chem.* **153**, 375-380.

Nelson, T. E., Johnson, J. Jr., Jantzen, E., and Kirkwood, S. (1969) Action pattern and specificity of an exo-β-(1→3)-D-glucanase from Basidiomycetes species QM 803. *J. Biol. Chem.* **244**, 5972-5980.

Obayashi, T., Yoshida, M., Mori, T., Goto, H., Yasuoka, A., Iwasaki, H., Teshima, H., Kohno, S., Horiuchi, A., Ito, A., Yamaguchi, K., Shimada, K., and Kawai, K. (1995) Plasma (1-3)-β-D-glucan measurement in diagnosis of invasive deep mycosis and fungal febrile episodes. *Lancet.* **345**, 17-20.

Ohkuma, T., Oyagiri, K., Ikekawa, T., and Tanaka, S. (1982) Augmentation of antitumor activity by combined cryo-destruction of sarcoma 180 and protein-bound polysaccharide, EA6, isolated from *Flammulina velutipes* (Curt. ex Fr.) Sing. in ICR mice., *J. Phrmacobio-Dyn.*, **5**, 439-444.

Ohga, S. (1992) Comparison of extracellular enzyme activities among different strains of *Lentinus edodes* grown on sawdust-based cultures in relationship to their fruiting abilities. *Mokuzai gakkaishi* **38**, 310-316.

Ohno, N., Iino, K., Suzuki, I., Oikawa, S., Sato, K., Miyazaki, T., and Yadomae, T. (1985) Neutral and acidic antitumor polysaccharides extracted from cultured fruit bodies of *Grifola frondosa*. *Chem. Pharm. Bull.* **33**, 1181-1186.

Ohno, N., Iino, K., Takeyama, T., Suzuki, I., Sato, K., Oikawa, S., Miyazaki, T., and Yadomae, T. (1985) Structural characterization and antitumor activity of the extracts from matted mycelium of cultured *Grifola frondosa*. *Chem. Pharm. Bull.* **33**, 3395-3401.

Ohno, N., Hayashi, M., Iino, K., Suzuki, I., Oikawa, S., Sato, K., Suzuki, Y., and Yadomae, T. (1986a) Effect of glucan on the antitumor activity on grifolan. *Chem. Pharm. Bull.* **34**, 2149-2154.

Ohno, N., Adachi, Y., Suzuki, I., Sato, K., Oikawa, S., and Yadomae, T. (1986b) Characterization of the antitumor glucan obtained from liquid-cultured *Grifola frondosa*. *Chem. Pharm. Bull.* **34**, 1709-1715.

Ohno, N., Suzuki, I., and Yadomae, T. (1986c) Structure and antitumor activity of a β-1, 3-glucan isolated from the culture filtrate of *Sclerotinina sclerotiorum* IFO 9395. *Chem. Pharm. Bull.* **34**, 1362-1365.

Okazaki, M., Adachi, Y., Ohno, N., and Yadomae, T. (1995) Structure-activity relationship of (1-3)-β-D-glucans in the induction of cytokine production from macrophages, *in vivo*. *Biol. Pharm. Bull.* **18**, 1320-1327.

Pitson, S. M., Seviour, R. J., and McDougall, B. M. (1993) Noncellulolytic fungal β-glucanases: their physiology and regulation. *Enzyme Microb. Technol.* **15**, 178-192.

Pitson, S. M., Seviour, R. J., McDougall, B. M., Woodward, J. R., and Stone, B. A. (1995) Purification and characterization of three extracellular (1→3)-β-D-glucan glucohydrolases from the filamentous fungus *Acremonium persicinum*. *Biochem. J.* **308**, 733-741.

Rose, W. C., Reed III, F. C., Siminoff, P., and Bradner, W. T. (1984) Immunotherapy of madison 109 lung carcinoma and other murine tumors using lentinan. *Cancer Res.* 44, 1368-1373.

Roslansky, P., and Novitsky, T. J. (1991) Sensitivity of *Limulus* amoebocyte lysate (LAL) to LAL-reactive glucans. *J. Clin. Microbiol.* **29**, 2477-2483.

Rylander, R., Persson, K., Goto, H., Yuasa, K., and Tanaka, S. (1992) Airborne beta-1, 3-glucan may be related to symptons un sick building. *Indoor environ.* **1**, 263-267.

Saito, H., Ohki, T., Takasuka, N., and Sasaki, T. (1977) A ^{13}C-N.M.R.-spectral study of a gel-forming, branched (1→3)-β-D-glucan, (lentinan) from *Lentinus edodes*, and its acid-degraded fraction. Structure, and dependence of conformation on the molecular weight. *Carbohydr. Res.* **58**, 293-305.

Saito, H., Ohki, T., and Sasaki, T. (1979) A ^{13}C-nuclear magnetic response study of polysaccharide gels. Molecular architecture in the gels consisting of fungal, branched (1→3)-β-D-glucans (lentinan and schizophyllan) as manifested by conformational changes induced by sodium hydroxide. *Carbohydr. Res.* **74**, 227-240.

Sakurai, T., Ohno, N., and Yadomae, T. (1994) Changes in immune mediators in mouse lung produced by administration of soluble (1-3)-β-D-glucan. *Biol. Pharm. Bull.* **17**, 617-622.

Sasaki, T., and Takasuka, N. (1976) Further study of the structure of lentinan, an anti-tumor polysaccharide from *Lentinus edodes*. *Carbohydr. Res.* **47**, 99-104.

Sasaki, T., Takasuka, N., Chihara, G., and Maeda, Y. Y. (1976) Antitumor activity of degraded products of lentinan: its correlation with molecular weight. *Gann* **67**, 191-195.

Somogyi, M. (1952) Notes on sugar determination. *J. Biol. Chem.*, **195**, 19-23.

Stone, B. A., and Clarke, A. E. (1992) In Chemistry and biology of (1-3)-β-glucans, LaTrobe University Press, Melbourne

Suga, T., Shiio, T., Maeda, Y. Y., and Chihara, G. (1984) Antitumor activity of lentinan in murine syngeneic and autochthonous hosts and its suppressive effect on 3-methylcholanthrene-indyced carcinogenesis. *Cancer Res.* **44**, 5132-5137.

Tabata, K., Itoh, W., Hirata, A., Sugawara, I., and Nori, S. (1990) Preparation of polyclonal antibodies to an anti-tumor (1-3)-β-D-glucan, Schizophyllan. *Agric. Biol. Chem.* **54**, 1953-1959.

Takada, K., Ohno, N., and Yadomae, T. (1994) Binding of lysozyme to lipopolysaccharide suppresses tumor necrosis factor production in vivo. *Infect. Immun.* **62**, 1171-1175.

Takahashi, M., Komuro, M., and Soutome, S. (1978) Purification and properties of β-1, 3-glucanase from *Lentinus lepideus*. *J. Ferment. Technol.* **56**, 499-505.

Takano, Y., Yagita, H., Iida, N., Hashimoto, H., Okumura, K., and Hirose, S. (1988) Relation between the level of IgG subclasses and infections in patients with systemic lupus erythematosus. *Int. Arch. Allergy Appl. Immunol.* **87**, 55-58.

Takeyama, T., Suzuki, I., Ohno, N., Oikawa, S., Sato, K., Ohsawa, M., and Yadomae, T. (1987) Host-mediated antitumor effect of grifolan NMF-5N, polysaccharide obtained from *Grifola frondosa. J. Pharmacobio-Dyn.* **10**, 644-651.

Tanaka, S., Aketagawa, J., Takahashi, S., Shibata, Y., Tsumuraya, Y., and Hashimoto, Y. (1991) Activation of limulus coagulation factor G by (1-3)-β-D-glucan. *Carbohydr. Res.* **218**, 167-174.

Tsukagoshi, S., Hashimoto, Y., Fujii, G., Nomoto, K., and Orita, K. (1984) Krestein (PSK). *Cancer Trear. Rev.* **11**, 131.

Wang, G., Zhang, J., Mizuno, T., Zhuand, C., Ito, H., Mayuzumi, H., Okamoto, H., and Li, L. (1993) Antitumor active polysaccharides from the Chinese mushroom *Songshan Lingzhi*, the fruiting body of *Ganoderma tsugae. Biosci. Biotech. Biochem.* **57**, 894-900.

Wasser, S. P., and Weis, A. L. (1999) Therapeutic effects of substances occurring in higher basidiomycetes mushroom: A modern perspective. *Crit. Rev. Immunol.* **19**, 65-96.

Wong, C. K., Leung, K. N., Fung, K. P., and Choy Y. M. (1994a) Immunomodulatory and anti-tumor polysaccharides from medicinal plants. *J. Int. Med. Res.* **22**, 299-312.

Wong, C. K., Leung, K. N., Fung, K. P., and Choy Y. M. (1994b) Effects of *Pseudostellaria heterophylla* on proliferation and differentiation of murine bone marrow cells. *Immunopharmacol. Immunotoxicol.* **16**, 71-84.

Wong, C. K., Leung, K. N., Fung, M. C., Fung, K. P., and Choy Y. M. (1994c) The induction of cytokine gene expression in murine peritoneal macrophages by *Pseudostellaria heterophylla. Immunopharmacol. Immunotoxicol.* **16**, 347-357.

Yamaguchi, Y., Yamashita, I., and Aoki, S. (1988) Studies on the preservation of Shii-take mushroom (*Lentinus edodes* (Bark) Sing.) at producer farm. *J. Jpn. Soci. Cold Preserv. Food* **14**, 59-62.

Yoshida, H.; Fujimoto, S., and Hayashi, J. (1991) Changes in the control of carbohydrates and organic acids in fruiting-bodies of maitake-mushroom (Grifolan frondosa (Fr.) S. F. Gray) during storage. *J. Japan Soc. Cold Preserva. Food* **17**, 45-52.

Zákány, J., Chihara, G., and Fachet, J. (1980a) Effect of lentinan on tumor growth in

murine allogeneic and syngeneic hosts. *Int. J. Cancer* **25**, 371-376.

Zákány, J., Chihara, G., and Fachet, J. (1980b) Effect of lentinan on the production of migration inhibitory factor induced by syngeneic tumor in mice. *Int. J. Cancer* **26**, 783-788.

Zákány, J., Jánossy, T., Németh, P., Chihara, G., Fachet, J., and Petri, G. (1983) Mechanism of the A/Ph. MC. S1 tumor graft rejection in syngeneic mice. *Gann* 74, 712-722.

Zhuang, C., Mizuno, T., Shimada, A., Ito, H., Suzuki, C., Mayuzumi, Y., Okamoto, H., Ma, Y., and Li, J. (1993) Antitumor protein-containing polysaccharides from a Chinese mushroom *Fengweigu* or *Houbitahe, Pleurotus sajor-coju* (Fr.) Sings. *Biosci. Biotech. Biochem.* 57, 901-906.

List of Publications

(1) Preparation and specificity of antibodies to an anti-tumor β-glucan, lentinan.

Biochemistry and Molecular Biology International, Vol. 39, No. 4, 679-685, 1996

(2) Preparation of antibody against antitumor β-glucan in *Grifola frondosa* and its application.

Food Science and Technology Research, Vol. 5, No. 4, 1999, in press

(3) Autolysis of lentinan, an antitumor polysaccharide, during storage of *Lentinus edodes*, Shiitake mushroom.

Journal of Agricultural and Food Chemistry, Vol. 47, No. 4, 1530-1532, 1999

(4) Influence of storage conditions on immunomodulating activities in *Lentinus edodes* (Berk.) Sing. (Agaricales s. l., Basidiomycetina).

International Journal of Medicinal Mushroom, Vol. 1, No. 3, 1999, in press

ACKNOWLEGEMENT

The author would like to express his grateful thanks to Professor Dr. Hironobu Tsuchida, Associate Professor Dr. Masashi Mizuno for kind guidance and various suggestions throughout the course of this study.

The author wishes to express his sincere gratitude to Professor Drs. Akira Shimizu and Youichi Kanazawa of Kobe University for their helpful advice and useful comments.

The author wishes to express thanks to Associate Professor Drs. Hitoshi Ashida and Kei-ichi Nomura of Kobe University for their helpful advice.

In this study, the author had many discussions with his colleagues, Mrs. Takashi Hashimoto, Akio Yamakawa, Shigenobu Tatsuoka and Yutaka Denpou. Their contributions to this study have been grate and the author acknowledges the important part played by them.

The author thanks Association Professor Dr. Hirofumi Terai of Kobe University for his kind helps.

It is my pleasure to acknowledge the supports and encouragement by the members of the Laboratory of Resource Science, Faculty of Agriculture, Kobe University.

The author is also grateful to his parents for the support while at school.