Preparation of Antibody against Antitumor \( \beta \)-Glucan in Grifola frondosa and Its Application

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Antibodies against an antitumor \( \beta \)-glucan purified from Grifola frondosa (GGF) were raised in the rabbit by subcutaneous immunization. Our antibodies reacted significantly with GGF by an ELISA inhibition assay. The antibodies did not recognize other polysaccharides such as laminarin and pustulan, but reacted somewhat with lentinian, whose structure is similar to GGF. It was demonstrated that GGF could be measured by ELISA using antibodies. In addition, the effects of the storage temperature on GGF content during storage were measured using our antibody. GGF content was 24.7 \( \mu \)g/g fresh weight (f.w.) at zero time storage, and little change occurred during storage of the mushroom for 7 days at 5°C. However, a drastic decrease to 1.4 \( \mu \)g/g f.w. occurred after 7 days of storage at 20°C. These results suggest that storage at low temperatures is desirable to maintain the quality of GGF.

Keywords: Immunomodulating effect, anti-tumor polysaccharides, ELISA, polyclonal antibody, Grifola frondosa

\( \beta \)-Glucan purified from the fruit body of Grifola frondosa (GGF) as an antitumor polysaccharide is composed of a backbone of \( \beta-(1 \rightarrow 6) \)-linked-D-glucose residues (Nanba et al., 1987). This \( \beta-(1 \rightarrow 6) \)-glucan possesses \( \beta-(1 \rightarrow 3) \) glucose side chains. Grifolan has been isolated and purified from liquid-cultured Grifola frondosa, and it has recently been reported that grifolan has strong host-mediate anti-tumor activity against various tumors. Grifolan administered \textit{in vivo} reportedly enhances immunoreactivity, including the activation of cytotoxic T cells and NK cells (Ohno et al., 1986a; b; Takeyama et al., 1987; Adachi et al., 1989). It also activates macrophages to release the cytokines IL-1, IL-6, tumor necrosis factor-\( \alpha \) (TNF-\( \alpha \)) and nitric oxide (NO) (Ohno et al., 1996; Hashimoto et al., 1997). Recent studies have demonstrated that murine macrophages stimulated with TNF-\( \alpha \) (Lorsbach et al., 1993) produce NO via expression of the inducible NOS gene (Xie et al., 1992), and the reactive nitrogen intermediates are thought to play a significant role in tumoricidal and microbicidal activities (Lowenstein & Snyder, 1992; Lorsbach et al., 1993). TNF-\( \alpha \) and NO have been noted as factors that show anti-tumor activity because they have direct cytotoxic activity on tumor cells and show a wide diversity of physiological activity in immune systems. Thus, Grifola frondosa seems to be an important functional food possessing anti-tumor activity. However, little has been reported as to how the content of GGF in Grifola frondosa is changed under storage conditions. Although analytical methods have improved considerably, it is still difficult to purify and quantify polysaccharides. Immunoassay, however, seems to be a simple and exact method that may make possible a more precise analysis of the glucan (Takano et al., 1988; Mizuno et al., 1996). Indeed, Adachi et al. (1994) reported the preparation of polyclonal antibodies to grifolan-BSA (bovine serum albumin) conjugate, and Hirata et al. (1994) reported the successful preparation of monoclonal antibodies against grifolan.

In this study, we planned to make use of enzyme-linked immunosorbent assay (ELISA) to easily and exactly quantify the GGF content in extracts from Grifola frondosa according to methods reported previously (Mizuno et al., 1996). This paper describes the preparation of polyclonal antibodies against GGF and their specificity to GGF compared with other polysaccharides. The change in GGF content during storage was also measured by an inhibition assay of ELISA using the anti-GGF antisera, and the TNF-\( \alpha \) and NO production from mouse peritoneal macrophages \textit{in vitro} was examined to determine whether GGF shows immunomodulating activity.

Materials and Methods

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

Materials GGF was purified from the fruit body of Grifola frondosa according to the methods of Nanba et al. (1987) and Chiha et al. (1969, 1970). Purified lentinian was kindly donated by Ajinomoto Co. Laminarin (\( \beta-1,3 \)-glucan) and pustulan (\( \beta-1,6 \)-glucan) were purchased from Sigma Chemical (St. Louis, MO) and Calbiochem (La Jolla, CA), respectively.

Preparation of peritoneal macrophages Peritoneal macrophages were isolated from mice (BALB/c, Japan SLC Co, Shizuoka) that had been injected intraperitoneally with 2 ml of 4.05% (w/v) fluid thioglycollate medium 3 days prior to peritoneal lavage with 10 ml of serum-free RPMI 1640 medium. The collected cells were washed with RPMI 1640 and cultured in RPMI 1640 containing 5% fetal calf serum at
a density of $0.5 \times 10^6$ cells/well. The cells were plated on a 24 flat-bottom well microculture plate and then incubated for 2 h at 37°C and 5% CO$_2$. After removing the nonadherent cells, the monolayered macrophages were stimulated with sample solution for 24 h at 37°C and 5% CO$_2$. At the end of the incubation, culture supernatant was collected by centrifugation at 2000×$g$ for 5 min.

**Measurement of TNF-α activity**  
TNF-α activity was measured by closely following the procedure described by Kerékgyártó et al. (1996). TNF-α in culture supernatants obtained from untreated or activated macrophages was measured by a biological assay using actinomycin D-treated L-929 mouse fibroblast cells. Recombinant murine TNF-α (Wako Pure Chemical Industries, Ltd., Osaka) was used as a standard. Briefly, L-929 cells were plated on a 96-well flat-bottom microculture plate at a density of $2 \times 10^4$ cells/ml in complete medium containing 4 μg/ml actinomycin D. Culture supernatant of peritoneal macrophages was then added to the plates. After 20 h incubation at 37°C, the remaining viable cells were fixed and stained with 0.1% crystal violet, and absorbance was measured at 570 nm.

**Nitrite assay**  
Macrophages were plated at a density of 1×10^5 cells/well in 24-well plates. After 24 h incubation, synthesis of NO was determined by an assay of culture supernatants for nitrite (NO$_2$), the stable reaction product of NO with molecular oxygen, as described by Stuehr and Nathan (1989). Briefly, 100 μM of Griess reagent was added to 100 μl of each supernatant in the 96-well plates and the absorbance was measured at 570 nm.

**Preparation of anti-GGF antibodies**  
GGF (0.5 mg) was dissolved in 1 ml of phosphate-buffered saline (PBS; pH 7.2). This solution was emulsified with an equal volume of Freund’s complete adjuvant. The emulsion was injected subcutaneously at 10 different sites on the back of the rabbit. A half volume of GGF of the first dosage was boosted again two weeks after the injection. After the boost, blood was collected to obtain anti-GGF antisera three times each week; the antisera were stored at −80°C until use. After measurement of the antibody titer, the rabbit was exsanguinated.

**Titre of the antibodies**  
The titer of the antisera was measured by ELISA as follows. The solution (100 μl) of antigen (GGF in PBS, 5 μg/ml) was placed in micro-titer wells (96 wells, Sumitomo Bakelite Co., Tokyo), and coated overnight at 4°C. All wells were then filled with PBS containing 1% skim milk for blocking and kept for 2 h 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 1:1000 to 1:100,000 diluted antisera was added to the micro-titer wells, and the wells were incubated for 1.5 h at 20°C. After washing three more times with PBS-Tween solution, 100 μl of 1:2000 dilute peroxidase-conjugated goat anti-rabbit IgG serum (Wako Pure Chemical Industries, Ltd.) was added to the wells and incubated for 1.5 h at 20°C. After frequent washing and coloration with H$_2$O$_2$-O-phenylenediamine (100 μl), absorbance at 492 nm was measured by an ELISA Auto Reader (Corona Electric Co., Ltd., Japan).

**Inhibition assay of ELISA**  
An inhibition assay of ELISA was used to confirm the specificity of the anti-GGF antibodies against some polysaccharides as described previously (Mizuno et al., 1996). The solution of antigen (GGF in PBS, 100 μg/ml) was coated in micro-titer wells overnight at 4°C. The antisera were incubated with the samples or various standard polysaccharides dissolved in PBS for 30 min. After blocking of the wells with skim milk and washing, the solution (100 μl) mixed with the antibodies and the samples were added to the wells. The plates were incubated for 1 h at 4°C and washed with PBS-Tween. Peroxidase-conjugated goat anti-rabbit IgG serum was added, and the plates were incubated for 1.5 h at 20°C. After washing and coloration with H$_2$O$_2$-O-phenylenediamine (100 μl), the absorbance at 492 nm was measured.

**GGF content in Grifola frondosa during storage**  
Grifola frondosa were packed in pored polyethylene bags (260 mm×380 mm, 0.03 mm thickness), and stored at 5°C or 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 until analysis. Each sample was homogenized with 20 volumes of distilled water. The homogenates were extracted in a 95°C water bath under reflux for 10 h, filtered, and then lyophilized. The lyophilized powders were dissolved in PBS and applied to an inhibition assay of ELISA.

**Results and Discussion**  
**Effect of GGF on TNF-α and NO production from macrophages**  
The molecular weight of GGF was determined to be ca. 2,000,000 by gel filtration on Sepharose CL-4B using the following standard pullulan (Showa Denko K.K., Tokyo): P-800(M.W.: 850,000), P-400 (380,000) and P-200 (186,000), and Blue Dextan (2,000,000) (data not shown). This molecular weight coincided with the data of Nanba et al. (1987). Though it is known that grifolan elicits the release of TNF-α and NO from macrophages (Ohno et al., 1996; Hashimoto et al., 1997), the release of these two factors with GGF has not yet been reported. Hence, we measured the effect of GGF on the production of TNF-α and NO from peritoneal macrophages. As shown in Figs. 1 and 2, GGF augmented the release of both factors from macrophages with a dose-dependency of 1 to 10 μg/ml. These results suggest that GGF has immunomodulating activity and also that it may have antitumor activity.

**Specificity of anti-GGF antisera**  
Anti-GGF antibodies were raised in a rabbit by immunization. The absorbance at 492 nm for the antisera as evaluated by ELISA is shown in Fig. 3. Prior to the infection the serum did not respond to GGF. Specificity of the anti-GGF antisera to some polysaccharides using the inhibition assay of ELISA is shown in Fig. 4. Although the antisera reacted somewhat with lentican, they recognized GGF more significantly. As the structure of lentican is similar to that of GGF, with the former having five β-(1→3)-glucose residues on each of the two β-(1→6)glucose side chains (Sasaki et al., 1976) and the latter having β-(1→3)glucose side chains on the β-(1→6)-glucose residues (Nanba et al., 1987), the anti-GGF antibody might react with lentican only at high concentrations. The molecular weights of GGF and lentican are 2,000,000 and 400,000, respectively, meaning that the specificity of this antibody against GGF is approximately a 5-fold
Fig. 1. Effect of GGF on TNF-α production in the culture supernatant from peritoneal macrophages.

Fig. 2. Effect of GGF on NO production in the culture supernatant from peritoneal macrophages.

molar ratio compared to lentilin when an ELISA inhibition assay of GGF and lentilin show the same values. On the other hand, the anti-GGF antisera did not recognize laminarin (a straight chain of β-1,3-glucan) or pullulan (a straight chain of β-1,6-glucan), indicating that the anti-GGF antisera may primarily recognize a certain core of the ratio of β-(1,6)-linked glucose to β-1,3-linked glucose. A linear calibration curve was obtained in the range of 0.5 to 10 μg/ml (Fig. 4). These results demonstrate that GGF content can be measured sufficiently by ELISA using the anti-GGF antibody prepared in the present study.

Change in the content of GGF in Grifola frondosa

The change in content of GGF in Grifola frondosa during storage at 5 and 20°C was calculated based on the results of

Fig. 3. Reactivity of the anti-GGF antisera as assessed by ELISA.

Fig. 4. Specificity of anti-GGF antibodies to polysaccharides. The horizontal bar shows the range of the linear relationship. (○) GGF, (●) pullulan, (■) laminarin, (▲) lentilin, (●) pullulan.

Fig. 5. Change in the GGF content of Grifola frondosa during storage. (●) 5°C; (○) 20°C.
the inhibition assay of ELISA. As shown in Fig. 5, the content of GGF immediately after harvest was at its highest, with a 24.7 μg/g fresh weight (f.w.), then decreased to 11.4 μg/g 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 *Grifola frondosa* decreased more significantly during storage at 20°C than at 5°C (data not shown). The rate of weight loss after 7 days during storage at 20°C and 5°C was approximately 8% and 3%, respectively, as compared with the fresh weight at zero time storage. In addition, the appearance of *Grifola frondosa* browned more at 20°C than at 5°C during storage. It has been reported that *Grifola frondosa* remained fresher longer at 5°C than at 10, 20 or 30°C, and no drastic changes in the freshness were observed at 1 or 5°C (Yoshida et al., 1991). According to Minamide et al. (1980), mushrooms such as *Lentinus edodes*, *Pholiota nameko*, *Agaricus bisporus*, *Pleurotus ostreatus*, and *Flammulina velutipes* remain edible longer in storage at 6°C than at 20°C. A low temperature (5°C) was reported to effectively maintain the content of lentinon, an antitumor polysaccharide in *Lentinus edodes* (Minato et al., 1999). These results suggest that low-temperature storage (such as 5°C) is desirable to maintain GGF content and the quality of *Grifola frondosa* as a functional food.

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References


