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Enzyme immunoassay system for estimating the ultrastructure of (1,6)-branched (1,3)-β-glucans

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Abstract

A sandwich-type enzyme immunoassay (EIA) system for quantifying branched (1,3)-β-glucans was established. A polyclonal antibody was purified with antigen-conjugated aminocellulofine and labelled with biotin to be used as the detection antibody. The reactivity of the antibody was restricted to only (1,6)-branched (1,3)-β-glucans. Molecular weight dependency of (1,6)-branched (1,3)-β-glucan in the reactivity was also observed. Alkaline-treated (1,6)-branched (1,3)-β-glucan which was reported to be a single helical conformer, showed a lower absorbance compared to the untreated triple helix conformer. The conformational alteration of the single helix to the triple helix was produced by heating for 15 min at 100°C. The results suggest that EIA has higher reactivity to the triple helical ultrastructure of (1,6)-branched (1,3)-β-glucans, and can be applied to estimate the conformational changes of (1,6)-branched (1,3)-β-glucans.

Keywords: (1,3)-β-glucans; Sandwich EIA; Helix conformation

1. Introduction

(1,3)-β-glucan is known as a potent immunostimulator and has significant augmenting effects on the host defense systems (Yadomae & Ohno, 1996). (1,6)-Branched (1,3)-β-glucans, such as sonifilan (SPG) and lentinan are used clinically (Fujimoto et al., 1988; Taguchi, Kaneko & Chihara, 1988). The physicochemical properties of (1,6)-branched (1,3)-β-glucans vary due to the differences in their branching ratios, molecular weight and ultrastructure (Yadomae et al., 1996). Biologically active (1,3)-β-glucans generally have gel-forming ability due to the formation of a single- or triple-helical conformation (Saito, Ohki & Sasaki, 1979; Ohno et al., 1986a). On alkali treatment the triple helix form of the (1,3)-β-glucan reverts to a random coil conformation (Ogawa, Watanabe, Tsurugi & Ono, 1972).

Neutralization of the glucan solution induces formation of a single helix (Ohno et al., 1986a). These conformational changes summarised in Fig. 1 are quite important for inducing several physiological phenomena such as activation of humoral host defense and macrophage function (Yanaki, Itoh & Tabata, 1986; Adachi, Ohno, Ohsawa, Sato, Oikawa & Yadomae, 1989; Nagi et al., 1993).

There have been some reports describing the detection of (1,3)-β-glucans in physiological sources (Nagi et al., 1993; Miura, Ohno, Aketagawa, Tamura, Tanaka & Yadomae, 1996). For instance, factor G from an amoebocyte lysate of horseshoe crab (*Limulus*) is highly sensitive to (1,3)-β-glucans (Tamura, Tanaka, Oda, Uemura, Aketagawa & Hashimoto, 1996). Factor G was reported to specifically bind to the linear moiety of (1,3)-β-glucans.

Owing to its high sensitivity, factor G could detect even spontaneously contaminated (1,3)-β-glucans in experimental fluids such as foetal bovine serum, cell culture medium, and buffer solution (Morita, Urashima, Tanaka, Aketagawa, Tamura & Kumazawa, 1996). We have shown that a *Limulus* assay is applicable for the measurement of the serum level of (1,3)-β-glucans in mice by using (1,3)-β-glucan-free fluid and experimental tools (Miura et al., 1996). However, other specific methods are needed to measure (1,3)-β-glucans. We have also reported that an antibody for grifolan, a (1,6)-branched type (1,3)-β-glucan from the edible mushroom, *Grifola frondosa*, showed cross-reactivity to *Sclerotinia sclerotiorum* glucan (SSG), Sonifilan prepared from schizophyllan (SPG), and lentinan (Adachi, Ohno & Yadomae, 1994). In the present study, we

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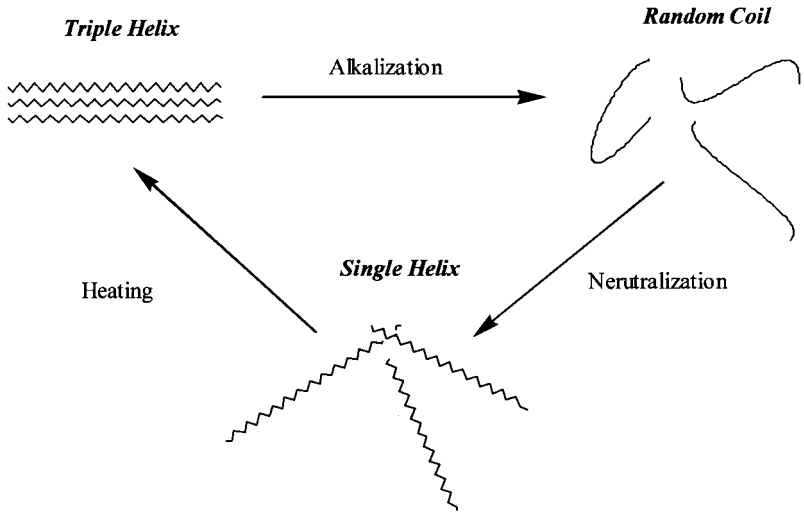

Fig. 1. Conformational changes for (1,3)-β-D-glucans.

established a sandwich enzyme immunoassay (EIA) system for detecting various (1,6)-branched (1,3)-β-glucans.

2. Materials and methods

2.1. Glucans

SPG was provided by Kaken Pharmaceutical Co. (Tokyo, Japan). SPG was reported to be a triple helical (1,3)-β-glucan (Norisuye, Yanaki & Fujita, 1980). GRN (grifolan) from *Grifola frondosa* and SSG, which is obtained from the culture broth of *Sclerotinia sclerotioum* IFO9395 were prepared as described previously (Ohno, Suzuki & Yadomae 1986b; and Ohno, Adachi, Suzuki, Sato, Oikawa & Yadomae, 1986c). Carboxymethylated curdlan (CM-curdlan) was prepared as described in Adachi et al. (1989). The degree of substitution of the carboxymethyl group per glucose residue was 0.2. Dextran T-500 was purchased from Pharmacia (Uppsala, Sweden).

2.2. Preparation of different molecular weight GRN

GRN powder was suspended in water at a concentration of 2.5 mg/ml and heated at 150°C for 15 min, 1, 3, 6, and 12 h in a seal-capped glass test tube with vigorous shaking. The molecular weights of the GRNs were estimated by gel-filtration using a Toyopearl HW-65F (Tosoh, Tokyo) as 500, 250, 21, 6.4 and $<$ 6.4 kD, respectively. It was reported that these heating conditions reduced the molecular weight of (1,3)-β-glucans (Adachi, Ohno, Yadomae, Suzuki, Ohsawa & Oikawa, 1990).

2.3. Preparation of conformationaly different GRNs

GRN was dissolved in 0.5 M NaOH at 5 mg/ml. An aliquot of 100 μl of the solution was neutralized by adding 0.5 M HCl and further diluted in 50 mM Tris–HCl buffer (pH 7.5) to 1 ml. The diluted solution was incubated for 15 min at room temperature, 40, 60, 80, or 100°C. These samples were used for the EIA after immediate cooling to room temperature.

2.4. Preparation of antibody

The antibody for GRN was prepared as described previously (Adachi et al., 1994). Briefly, GRN conjugated with bovine serum albumin (BSA) was dissolved in phosphate-buffered saline (PBS) and emulsified with an equal volume of complete Freund's adjuvant or incomplete adjuvant (Difco Laboratories, Detroit, MI, USA), and injected subcutaneously into a Japanese white rabbit (Saitama Laboratory Animals, Saitama, Japan). A booster injection was administered after a 2-week interval. Serum was collected at day 42 after the first immunization. The antibody fraction against GRN was isolated using a GRN-conjugated aminocellulofine affinity gel (Seikagaku Corp., Tokyo). The biotinylation of anti-GRN antibody was performed by mixing the antibody with NHS-LC-biotin (Pierce, Rockford, IL) in bicarbonate buffer (pH8.4).

2.5. Sandwich EIA

A 96 well plastic EIA plate (H-type Sumilon, Tokyo, Japan) was coated using a 50 μl portion of anti-GRN serum (diluted to 1/2500 with bicarbonate buffer, pH9.6) and incubated for 2 h at 37°C. The unbound antibody was washed away with PBS containing 0.05% Tween-20 (PBST). After washing, the wells were incubated with PBS containing 1% BSA (BPBS) for 40 min at 37°C. After incubating the plate with BPBS, various concentrations of polysaccharides dissolved in BPBS were added to each well, and the plate was further incubated for 40 min at 37°C. The plate was washed three times with PBST; biotinyl GRN antibody (diluted to 1/500 with BPBS) was then placed in the wells, and the plate was incubated for

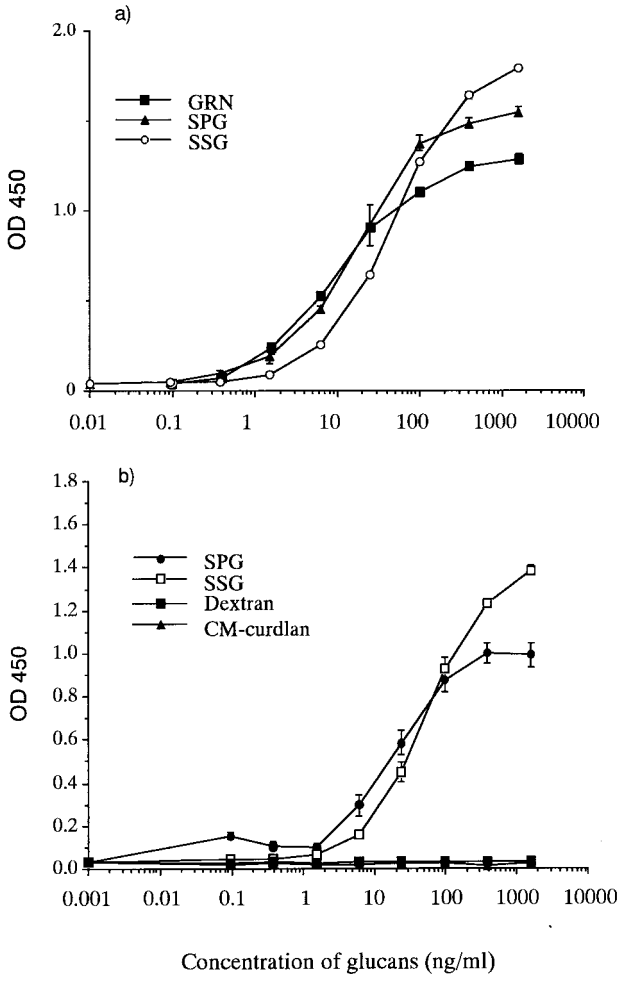

Fig. 2. Reactivity to primary structurally different glucans. Various glucans were subjected to the EIA. GRN, SPG, and SSG are (1,6)-branched (1,3)-β-D-glucans with branching ratios [(1,6)/(1,3)] of 1/3, 1/3 and 1/2, respectively. Dextran is a (1,6)-α-D-glucan. CM-curdlan is a carboxymethylated (1,3)-β-D-glucan. The ratio of carboxymethyl group to glucose residue is 0.2.

40 min at 37°C. After incubation, the wells were washed three times with PBST, and peroxidase-conjugated streptavidin (Vector, Burlingame, CA) in BPBS was added to the wells. The plate was then incubated for 20 min at room temperature. The enzyme reaction was terminated by adding 50 μl 1 M H_3PO_4, and the absorbance of the resulting solution was measured at 450 nm using a microplate reader (Corona MTP-32, Katsuta, Japan).

3. Results

3.1. Reactivity of various glucans in the sandwich EIA for GRN

In order to assess the reactivity of β-glucans, several structurally related (1,3)-β-glucans were used for the EIA assay. Various concentrations of glucans ranging from 0.01 to 1500 ng/ml were placed in duplicate wells of the microplate. As shown in Fig. 2(a), the reactivity of this EIA system was not restricted to grifolan, but also extended to SSG and SPG. These (1,3)-β-glucans are known to possess common structural properties, i.e., a (1,6)-monoglucosyl branch, high molecular weight more than 450 kD, and helix conformation (Norisuye et al., 1980; Ohno et al., 1986b; Adachi et al., 1990). To identify which structural properties are important for the reactivity, an α-glucan (dextran T-500) and a linear (1,3)-β-glucan (CM-curdlan) were used in the assay. Since curdlan could not be dissolved in water easily, a water–soluble derivative, carboxymethylated curdlan (CM-curdlan) was used instead of curdlan. As shown in Fig. 2(b), dextran and CM-curdlan were not detected by this EIA system, although SSG and SPG were detected.

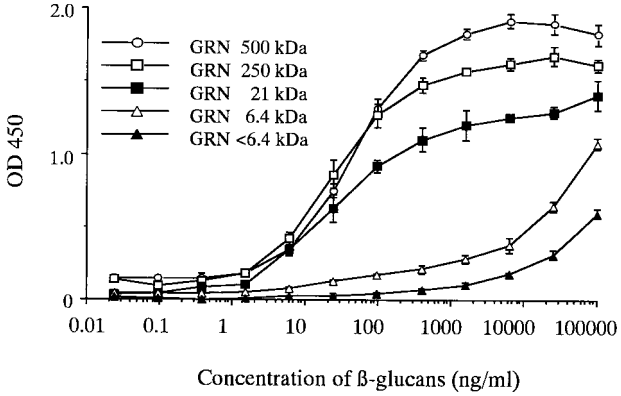

Fig. 3. Effect of molecular weight of (1,3)-β-glucan on reactivity in the EIA. GRN fragments possessing various molecular weights as indicated were subjected to the EIA.

3.2. Effect of molecular weight of (1,3)-β-glucan on the reactivity of the EIA

Molecular weight dependency on this reaction was also examined using heat-degraded GRN. The reactivity of the higher molecular weight fraction (more than 21 kD) showed higher reactivity than the lower molecular weight fraction (Fig. 3). The reactivity of this EIA seemed to be dependent on the molecular weight of (1,3)-β-glucans.

3.3. Conformation dependency on the reactivity of the sandwich EIA

The reactivity of different conformers of (1,3)-β-glucans was also examined. Alkaline-treated and neutralized GRNs showed lower reactivity on this EIA, and the reactivity of the neutralized GRNs was increased by heating (Fig. 4). It has been reported that the alkaline treatment of the triple-helical conformer changes it to the single helical form of (1,3)-β-glucan (Ohno et al., 1986a; Ohno, Adachi, Ohsawa, Sato, Oikawa & Yadomae, 1987). However, the conversion of the single helix to the triple helix conformation has been unclear. The present result suggests that the heating of the

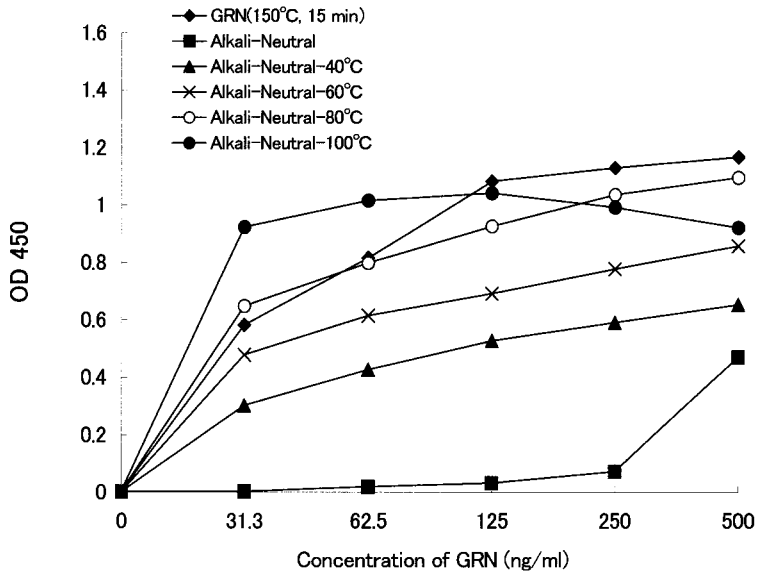

Fig. 4. Effect of conformation of (1,3)-β-glucans on reactivity in the EIA. The various GRN preparations described in materials and methods were subjected to EIA. GRN dissolved in water by heating at 150°C for 15 min was used as a standard.

single helical (1,3)-β-glucan may affect the conformational change and that the reactivity of this sandwich EIA system depends on the ultrastructure of the (1,3)-β-glucans.

4. Discussion

We have examined the reaction specificity of a newly established EIA for (1,3)-β-glucans. The results demonstrated that this EIA system had specificity only for (1,6)-branched (1,3)-β-glucans and that the EIA allowed us to measure the branched type (1,3)-β-glucan content. Our findings are in agreement with the results of our previous study using a competitive EIA system (Adachi et al., 1994). In that study, we tested 21 kinds of polysaccharides and oligosaccharides to determine the antigen specificity to anti-GRN antibody by using a competitive assay probed with biotinyl-GRN. SPG and lentinan are typical branched (1,3)-β-glucans, and are used clinically as anticancer medicines (Fujimoto et al., 1988; Taguchi et al., 1988). It was reported that branched type (1,3)-β-glucans are ubiquitous in the cell wall of fungi including *Candida albicans* (Duffus, Levi & Manners, 1982). It is thus important to detect branched types of (1,3)-β-glucans for evaluating the effect of anticancer drugs and for the diagnoses of fungal infections.

The reaction specificity in our new EIA was not restricted to only a primary structure, but extended also to the ultrastructure of (1,3)-β-glucans. Various molecular weight versions of GRN were prepared by heat denaturation at 150°C as reported earlier (Adachi et al., 1990). The reactivity of EIA was strongly dependent on the molecular weight of GRN. It is generally accepted that the triple helical conformer of (1,3)-β-glucans at higher molecular weight can be changed to the random coil state by reducing the molecular weight (Adachi et al., 1990), since short fragments of (1,3)-β-glucan chain were insufficient to cross-link to other glucan chains. The two kinds of antibody used in the new assay (i.e., capture and detection antibodies), have substantially equal specificity to branched (1,3)-β-glucans. If the (1,3)-β-glucan captured on the assay plate had a large amount of epitopes which would be the binding site for a biotinylated detection antibody, a number of detection antibodies can remain on the glucan chains. It was therefore expected that the highly cross-linked, i.e., triple helical branched (1,3)-β-glucans provide many binding sites for the detection antibody. To confirm this, we dissolved GRN in 0.5 M sodium hydroxide and subsequently neutralized the solution to produce the single helical conformation, as reported elsewhere (Ohno et al., 1986b). The alkaline-treated GRN showed less reactivity on the EIA. Moreover, to test the possibility that alkaline treatment made the glucan chain too short to bind to the antibody, the neutralized solution was heated at 40–100°C to reconstruct the ultrastructure. The heated product showed increased reactivity, comparable to that of the150°C-treated GRN, which is reported to show the triple helical conformation (Adachi et al., 1990). These results clearly demonstrated that the alteration of the ultrastructure of (1,3)-β-glucans influences the sensitivity of this sandwich EIA, and that triple helical branched (1,3)-β-glucans with high molecular weight are most reactive to this EIA system. Nagi et al. reported that the conversion of single helical SPG to the triple helix required 7 days at 4°C (Nagi et al., 1993). However, the present study demonstrated that heat treatment for 15 min at 100°C of a single-helical glucan at pH7.5 could induce annealing to form the triple helical conformation. This heat treatment is useful to estimate the triple helix content in the glucan solution. It has been

clarified that the ultrastructure of (1,3)-β-glucans is critical in the induction of biological activities such as macrophage activation (Ohno, Hashimoto, Adachi & Yadomae, 1996) and complement activation (Suzuki, Ohno, Saito & Yadomae, 1992). Therefore, the estimation of the ultrastructure of glucan solutions is quite important to predict the biological effect of various (1,3)-β-glucans.

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