Change of Biological Activities of (1→3)-β-D-Glucan from *Grifola frondosa* upon Molecular Weight Reduction by Heat Treatment

Yoshiyuki ADACHI,[a] Naohito OHNO,[a] Masumi OHSAWA,[b] Shozo OIKAWA,[b] and Toshiro YADOMAE*,[a]

Laboratory of Immunopharmacology of Microbial Products, Tokyo College of Pharmacy,[a] Horinouchi, Hachioji, Tokyo 192-03, Japan and Nippon Beet Sugar Mfg. Co., Ltd.,[b] Kyobashi, Chuo-ku, Tokyo 104, Japan. *Received August 10, 1989*

Changes of biological activities manifested by (1→6)-branched (1→3)-β-D-glucans of various molecular weights obtained by heat treatment of the corresponding intact β-glucan at 150 °C (HD-LE) were examined. The activities assessed in this study were as follows: an antitumor activity, activation of alternative complement pathway, glucose consumption by macrophages, macrophage-mediated lysosomal enzyme activity in culture supernatant and cell lysate, interleukin-1 (IL-1) activity, and adjuvant activity. HD-LE could be classified into three groups: 1) HD-LE 0 h (MW 800000) which activated all of the biological activities tested, 2) HD-LE 0.5 and 3 h (MW 250000 and 21000) which lacked or exhibited low levels of activities such as activation of alternative complement pathway and lysosomal enzyme secretion, 3) HD-LE 6 h (MW 6400) which only activated glucose consumption and synthesis of lysosomal enzyme. These results suggest that an antitumor glucan is not always a multiple enhancer of host defense mechanisms and that a large molecular weight is required to augment multiple immunological activities.

Keywords *Grifola frondosa*; (1→6)-branched (1→3)-β-D-glucan; molecular weight; antitumor activity; macrophage; complement; adjuvant activity

Immunological activities of several (1→3)-β-D-glucans have been extensively examined by many investigators.[1] The glucans directly stimulate macrophages or monocytes to secrete interleukin-1[2] (IL-1). IL-1 is thought to be important to induce subsequent activities such as augmentation of sensitivity to interleukin-2 on helper T lymphocytes,[3] induction of cytotoxic T cells,[4] enhancement of adjuvant activity.[5] Further, the ability of the (1→3)-β-D-glucans to trigger alternative complement pathway is assumed to be important in the first step of host defense mechanisms.[6] These results demonstrated that the (1→3)-D-glucans are broad-spectrum enhancers of host immune systems.

Most of the (1→3)-β-D-glucans have also been shown to possess antitumor activities. However, the relationships between antitumor activities and stimulation of immune systems by the (1→3)-β-D-glucans have not been clarified sufficiently. Comparative study of immunopharmacological activities induced with antitumor-active and -inactive (1→3)-β-D-glucans will be one strategy to resolve this problem. From the relationship between structure and antitumor activity, ultrastructure including triple-helix conformation has been considered to be important. Molecular weight (MW) is also an important factor for antitumor activity, because the (1→6)- branched (1→3)-β-D-glucans such as lentinan, schizophyllan, and grifolan could induce antitumor activity when they had molecular weights of more than about $1—4 \times 10^4$.[7]

We previously reported that heat treatment (above 150 °C) of branched (1→3)-β-D-glucans in solution is a useful method to reduce the molecular weight without alteration of the primary structure, and that the reduction of molecular weight changed the ordered conformation of the parent glucan to a random-coiled structure (submitted for publication). Therefore, it seemed interesting to examine whether the antitumor activity would change concomitantly, and to compare the relationship between antitumor activity and immunomodulating activities. In this report, we examined various biological activities of (1→6)-branched (1→3)-β-D-glucans of various molecular weights prepared by heat-treatment of LELFD (HD-LE) from *Grifola frondosa*.

Materials and Methods

Mice Male 6- to 8-week-old ICR mice were obtained from Shizuoka Agricultural Cooperative Association for Laboratory Animals, Shizuoka. The mice were held under specific pathogen free conditions.

Reagents Zymosan A (zymosan), lipopolysaccharide (LPS) obtained from *E. coli* 0127 : B8 by phenol extraction, *p*-nitrophenyl-β-D-glucuronide and ethylene glycol bis-amino tetraacetate (EGTA) were purchased from Sigma (St. Louis, MO, U.S.A.). Ethylenediamine tetraacetate (EDTA) was obtained from Dojin Laboratories, Kumamoto. Phytohemagglutinin-P (PHA) was purchased from Seikagaku Kogyo Co., Ltd., Tokyo. Glucose B-test Wako and β-D-glucuronidase from calf liver (EC 3.2.1.31) were purchased from Wako Chemical Industry.

Preparation of LELFD The polysaccharide fraction was prepared from liquid-cultured mycelium of *Grifola frondosa* as described previously.[8] Briefly, *G. frondosa* var. *Tokachiana* was grown in 100 ml of medium containing glucose (2.0%), polypeptone (0.6%), cane sugar (2.0%) and soybean oil (0.1%), pH 4.5, with reciprocal shaking at 25 °C for 14 d. The mycelium was separated from the filtrate by filtration, and was incubated in 0.5% citrate buffer (pH 4.0) containing 5% D-glucose at 25 °C. After incubation for 6 d, the mixture was centrifuged and the supernatant solution was diluted with 1 vol. of ethanol. An aqueous solution of the resulting precipitate was diluted with 1 vol. of ethanol, and the precipitate was collected, dried, and used as LELFD. LELFD was dissolved in 8 M urea, and the solution was dialyzed against water and then lyophilized.

Heat Treatment of LELFD LELFD was suspended in distilled water (2.5 mg/ml, 10 ml) and heated for various times at 150 °C using glass tubes with screw caps in an aluminium block heater. The products heated for 0, 0.5, 1.3, 2, 3, 6, 9, and 12 h were designated as HD-LE 0 h, HD-LE 0.5 h, HD-LE 1.3 h, HD-LE 2 h, HD-LE 3 h, HD-LE 6 h, HD-LE 9 h, and HD-LE 12 h, respectively.

Assay of Antitumor Activity Sarcoma 180 cells were maintained serially in the ascites form by weekly passage in ICR mice. The cells (5×10^6) were inoculated subcutaneously into the right groin of ICR mice. Each glucan fraction was dissolved in saline and administered intraperitoneally at 7 d after tumor inoculation. After 5 weeks, the mice were killed and the tumors were weighed. The antitumor activity was assessed from the percent inhibition, which was calculated as: [1 − (average tumor weight of the treated group/average tumor weight of the control group)] × 100 (%).

Activation of Alternative Complement Pathway Freshly prepared samples of human whole serum provided sources of complement. For assay, samples were dissolved in gelatin veronal buffer (GVB) containing 0.05 M EGTA and 0.01 M magnesium (EGTA–GVB–Mg^{2+}). Then, 0.1 ml of the sample was incubated with the complement solution (0.4 ml) at 37 °C for 1 h, and diluted with 0.01 M EGTA–GVB–Mg^{2+}. The solution (0.8 ml) was

mixed with 0.2 ml of rabbit red blood cells (rabbit-RBC, 5×10^7/ml), and incubated at 37 °C for 1 h. A 2.0 ml portion of GVB containing 0.01 M EDTA (EDTA–GVB) was added to the solution to block further complement activation. After centrifugation, the optical density of the supernatant was read at 414 nm.

Macrophage Culture Resident peritoneal macrophages from ICR mice were isolated from the peritoneal cavity by lavage with 2 washes of 5—6 ml of Hanks' balanced salt solution (HBSS) (Nissui Seiyaku Co., Ltd., Tokyo, Japan), 2 times. The recovered lavage fluid was centrifuged at 300 *g* for 5 min to pellet the cells. The pellet was washed twice and resuspended in RPMI 1640 (Nissui Seiyaku Co., Ltd., Tokyo, Japan) containing 1% heat-inactivated fetal calf serum (FCS) (Boehringer Mannheim, FRG), 5 mM *N'*-hydroxyethylpiperazine-*N'*-2-ethansulfonate (HEPES) (Sigma Chemical Co., St. Louis, MO, U.S.A.), 100 U/ml penicillin, 100 μg/ml streptomycin (Meiji Seika Kaisha, Ltd., Tokyo, Japan). The cells were cultured in 24-well or 96-well flat-bottomed plates (Sumitomo Bakelite Co., Ltd., Japan) at 2×10^6 per well (24 well) or 2×10^5 per well (96 well) in 1000 μl or 100 μl of culture medium respectively. To ensure adherence, cells were cultured for at least 2 h before washing, and washed twice with 500 or 50 μl/well of fresh medium. Obtained macrophages were cultured for 48 or 72 h in a humidified 5% CO_2 incubator at 37 °C with 500 μg/ml of HD-LE in RPMI 1640 medium containing 5% FCS, 5 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin. For IL-1 assay, culture supernatant from a 24-well culture was filtered through a syringe filter unit (0.20 μm, Corning), and macrophage lysate was prepared by repeating freeze and thawing (3 times) followed by filtration through the syringe filter unit.

Glucose Consumption Activity Glucose remaining in the macrophage culture supernatant was measured by the use of Glucose B-test Wako. The supernatants (10 μl) obtained from the macrophage culture sampled for 72 h at 37 °C were incubated with 1.5 ml of color reagent for 20 min at 37 °C. The optical density at 505 nm of the solution was measured and remaining glucose was determined from a calibration curve with standard glucose solution. The results were expressed as percent glucose consumption, calculated from the following formula: [1 −(glucose content in culture medium cultured with macrophages/glucose content in culture medium without macrophages)] × 100.

Lysosomal Enzyme Assay The activity of β-D-glucuronidase in the culture supernatant or cell lysate was measured by using *p*-nitrophenyl-β-D-glucuronide as a substrate. The culture was carried out for 72 h at 37 °C in 200 μl/well of RPMI 1640 containing 5% FCS. After the incubation, 100 μl of the culture supernatant or macrophage lysate prepared with 10 μl of 0.1% Triton X-100 was mixed with 200 μl of 6 M *p*-nitrophenyl-β-D-glucuronide in 0.1 M citrate buffer (pH 5.0), and incubated for 2 h at 37 °C. The reaction was terminated by adding 1 ml of 0.2 M borate buffer (pH 9.8). The *p*-nitrophenol released by the enzyme-dependent hydrolysis of the substrate was quantified spectrophotometrically by measuring optical density at 405 nm. The enzyme activities were expressed in units assessed by a simultaneous experiment with standard β-D-glucuronidase from calf liver.

IL-1 Assay IL-1 activity of supernatants or macrophage lysates was assayed in terms of the ability to stimulate murine thymocyte proliferation. Thymuses from C3H/HeJ mice were gently teased and filtered through a wire mesh to remove debris and aggregates. The cells were resuspended in RPMI 1640 with 5% FCS and with or without various additives as indicated. Thymocytes (1×10^6) were cultured for 72 h in a flat-bottomed 96-well culture plate. Cultures were exposed for the final 12 h of incubation to 0.5 μCi of ^{3}H-TdR (ICN Biomedical Inc., CA, U.S.A.). The cells were collected on glass fiber filters with an automatic harvester. The filters were dried, added to scintillation fluid, and counted in a scintillation counter. The results are expressed in mean cpm of ^{3}H-TdR taken up by triplicate cultures.

Plaque-Forming Cell (PFC) Assay The PFC response to sheep red blood cell (SRBC) was determined by the technique of Cunningham and Szenberg.[9]

Results and Discussion

Examination of Antitumor Activity of HD-LE The antitumor effects of heat-treated LELFD (HD-LE) were examined in the sarcoma 180-ICR mice system. Physicochemical properties including molecular weight of HD-LE are listed in Table I. As shown in Table II, significant antitumor effects were observed on administration of HD-LE 0 h, 0.5 h, 1.3 h, 2 h, and 3 h. HD-LE 6 h showed 45% inhibition of tumor growth, although the value was not statistically significant. The glucans of lower molecular weight than 21000 exhibited decreased growth-inhibitory effect on sarcoma 180 transplanted in mice. These results indicated that heat treatment more than for 3 h at 150 °C reduced their antitumor effect and that the critical molecular weight for the antitumor effect was around 2×10^4 which is similar to those in the cases of other antitumor glucans.[7] To examine the following biological activities, HD-LE 0 h, 0.5 h, 3 h, or 6 h as selected as antitumor-active or inactive samples.

TABLE I. Physicochemical Properties of HD-LE

Sample[a]		Molecular weight[b] ($\times 10^3$)	Relative content[c] of ordered structure
HD-LE	0 h	800	101.6
	0.5 h	250	82.1
	1.3 h	110	77.2
	2 h	40	71.4
	3 h	21	50.0
	6 h	6.4	32.5
	9 h	<6.4	23.0
	12 h	<6.4	33.2
GRN LE		500	100.0

a) HD-LE was prepared from LELFD by heat treatment for the indicated time at 150 °C. *b*) Each molecular weight was determined by gel-filtration eluted with 0.3 M NaOH. *c*) The content of ordered structure was assessed by measuring the fluorescence intensity of glucan–aniline blue complex in 0.1 M NaOH.

TABLE II. Antitumor Activity against Sarcoma 180 of HD-LEs[a]

Sample		Dose × 1 (μg/mouse)	Tumor weight[b] (g, mean ± S.D.)	Inhibition ratio (%)[c]	Complete regression[d]
HD-LE	0 h	1000	0.10 ± 0.15[d]	98	5/10
	0.5 h	1000	0.05 ± 0.11[d]	99	6/9
	1.3 h	1000	0.08 ± 0.27[d]	98	5/10
	2 h	1000	0.67 ± 1.59[d]	97	6/10
	3 h	1000	0.01 ± 0.05[d]	>99	9/10
	6 h	1000	2.81 ± 3.14	45	0/10
	9 h	1000	4.14 ± 3.38	19	0/10
	12 h	1000	5.41 ± 4.67	−6	0/10
GRN LE		1000	0.08 ± 0.11[d]	98	3/10
Nil			5.10 ± 3.27		0/22

a) Sarcoma 180 cells (5×10^6) were inoculated s.c. (day 0). Each sample was administered i.p. on day 7 as a saline solution. *b*) The significance was evaluated by means of Student's *t* test against the untreated group. *c*) Determined at day 35 after tumor inoculation. *d*) $p < 0.001$.

Examination of the Ability to Activate Alternative Complement Pathway by HD-LE The ability of HD-LE to trigger the alternative complement pathway was compared in each molecular weight fraction using unsensitized rabbit-RBC and human whole serum as a complement. As shown in Fig. 1, preincubation of the serum with 5 or 10 mg/ml of GRN LE[8] and HD-LE 0 h consistently decreased the residual hemolytic activity as well as did zymosan at the concentrations of 2.5 or 5 mg/ml. In contrast, pretreatment of the serum with other HD-LEs did not inhibit the hemolysis significantly, except that HD-LE 0.5 h (10 mg/ml) showed approximately 50% hemolytic activity with 0.5 ml of treated serum. These results indicate that lower-molecular-weight glucans such as HD-LE 3 h, 6 h, and 12 h have little or no ability to activate the alternative complement pathway under these conditions.

Examination of the Activating Effect of HD-LEs on

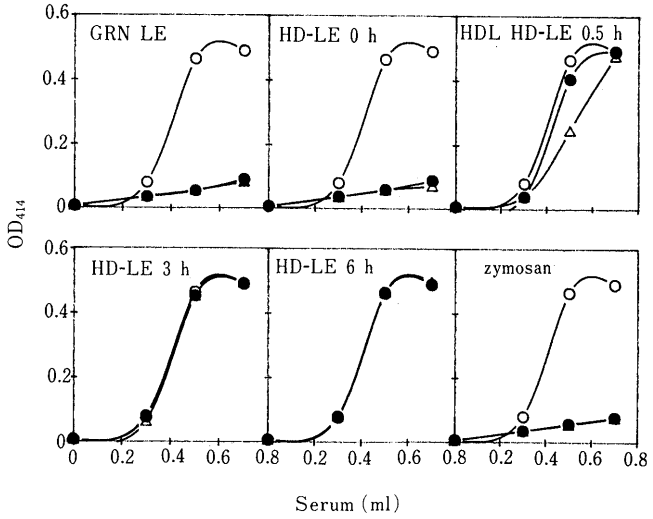

Fig. 1. Activation of the Alternative Complement Pathway by HD-LE

Human sera pretreated with indicated samples at the dose of 0 mg/ml (○), 5 mg/ml (●) and 10 mg/ml (△) for HD-LE and GRN LE, 2.5 mg/ml (●) and 5 mg/ml (△) for zymosan were diluted with 0.01 M EGTA–GVB–Mg^{2+} and added to rabbit-RBC. The degrees of hemolysis by the treated sera were compared in terms of the absorbance at 414 nm of the supernatant.

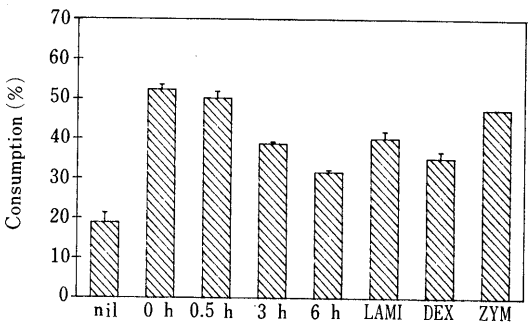

Fig. 2. Effect of HD-LE on Glucose Consumption by Macrophages

The glucose content in culture supernatant obtained from the wells after incubation for 72 h at 37 °C with each sample was assessed by using a kit (Glucose B-test Wako). The macrophages (2×10^5/well) were cultured in 200 μl of medium with various additives. The concentration of the soluble glucans was 500 μg/ml. LAMI, DEX, and ZYM represent samples of laminarin, dextran T-500, and zymosan (1×10^7 particles/ml), respectively.

Mouse Peritoneal Macrophages *in Vitro* Since 500 μg/ml of GRN LE, purified glucan from LELFD,[8] sufficiently enhanced macrophage functions in preliminary experiments (unpublished data), we used this concentration for the following assay. The effect of HD-LE on macrophage activation was first evaluated by measuring the glucose consumption by resident peritoneal macrophages stimulated *in vitro* with HD-LE (500 μg/ml). As shown in Fig. 2, all of the macrophages stimulated with samples used in this assay consumed larger amounts of glucose than did unstimulated macrophages. The consumption of glucose by addition of HD-LE 0 h and 0.5 h was around 50%. The extent of the consumption with HD-LE 3 h was less than that with HD-LE 0 h, and HD-LE 0.5 h, and that in the case of HD-LE 6 h was comparable to dextran T-500. These results suggested that molecular size is one of the critical factors to enhance glucose consumption of the peritoneal macrophages.

The effect of incubation with HD-LE on the release of lysosomal enzyme was examined by using resident peritoneal macrophages. The macrophages were cultured with

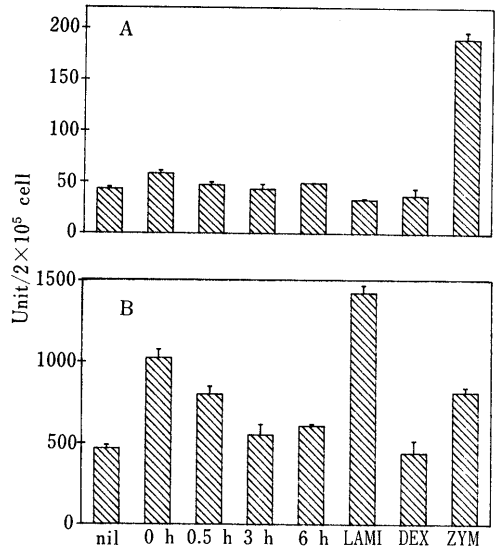

Fig. 3. Lysosomal Enzyme Production by Macrophages Incubated with HD-LE

The production of β-D-glucuronidase by macrophages cultured for 72 h at 37 °C with each sample was determined by measuring the release of *p*-nitrophenol from *p*-nitrophenyl β-D-glucuronide in culture supernatant and cell lysate. The culture condition were the same as in Fig. 2.

500 μg/ml of HD-LEs under exactly the same conditions as in the glucose-consumption assay. After the incubation, the β-D-glucuronidase activity in culture supernatants and macrophage lysates was measured by using *p*-nitrophenyl-β-D-glucuronide as a substrate. Besed on secreted enzyme in the supernatants, HD-LE 0 h and zymosan significantly enhanced the release of the enzyme ($p<0.01$ and $p<0.001$, respectively) (Fig. 3 A). These results substantially coincide with those of Schorlemmer *et al.*[10] who described the importance of activated complement components in macrophage activation, leading to the release of lysosomal enzymes. However, degree of the enzyme activity induced by HD-LE 0 h was rather less than that by zymosan. This might have resulted from the morphology of the samples, because HD-LE 0 h formed a gel, whereas zymosan formed particles. In the case of intracellular β-D-glucuronidase activity (Fig. 3B), cultivation with all of the glucans except for dextran T-500 increased the enzyme activity. However, some of the samples such as HD-LE 0 h, 0.5 h, laminarin, and zymosan strongly enhanced synthesis of the enzyme. The intracellular enzyme synthesized by stimulation with HD-LE 0 h was larger than that with zymosan. This result is similar to other findings by other workers[11] demonstrating that (1→3)-β-D-glucans were more effective to augment the enzyme synthesis than zymosan.

The effect of HD-LE on production of IL-1 by resident peritoneal macrophages *in vitro* was evaluated (Fig. 4). Enhancement of IL-1 production in the culture supernatant was observed by macrophages treated with HD-LE 0 h, 0.5 h, and 3 h. No increase of incorporation of ^{3}H-TdR into thymocytes took place in the absence of PHA (data not shown). The extent of IL-1 activity decreased with the reduction of molecular weight. In the case of IL-1 production in cell lysates, an augmentative effect was observed with HD-LE 0 h and 0.5 h. Incubation with HD-LE 6 h did not stimulate IL-1 production in the culture supernatant or cell lysate. These results indicated that HD-LE which

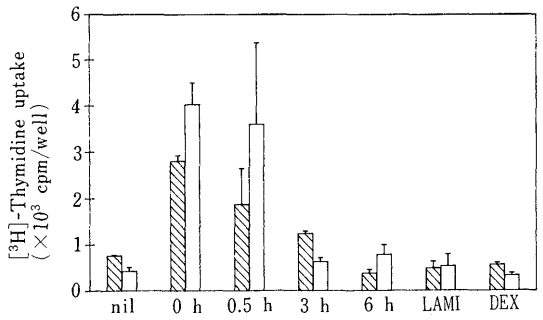

Fig. 4. IL-1 Activity of the Macrophages Stimulated with HD-LE

IL-1 activity in culture supernatant (hatched columns) and cell lysate (open columns) was assessed in terms of the incorporation of ^{3}H-TdR into thymocytes cultured with several preparations and PHA. Macrophages (2×10^6/ml, 1 ml) were incubated with various glucans at the concentration of 500 μg/ml. The thymocyte culture was carried out for 72 h and ^{3}H-TdR was added 12 h before the termination.

TABLE III. Adjuvant Effect of HD-LE on Antibody Response to SRBC[a]

Sample	Dose × 1 (μg/mouse)	Anti-SRBC PFC/spleen (mean ± S.D.) IgM	IgG
HD-LE 0 h	1000	3811 ± 1101	12618 ± 5126
0.5 h	1000	10516 ± 7694	19098 ± 10810
3 h	1000	2508 ± 1641	8288 ± 5066
6 h	1000	2482 ± 2100	4563 ± 3503
LPS	10	6534 ± 1946	16552 ± 7184
Nil (Saline)		1363 ± 865	3749 ± 991

a) Each sample and SRBC (1×10^7) were administered i.p. to ICR mice (4 mice/group) simultaneously. On day 7 after the administration, the number of PFC from spleen was counted. Response of IgG to SRBC was determined as the difference between PFC with and without anti-mouse IgG antibody.

possessed a molecular weight of not less than 250000 could induce a significant amount of IL-1, whereas even at a molecular weight of around 21000, HD-LE stimulated IL-1 about 1.6-fold over the control (significance $p < 0.001$). LTN and SPG were also reported to stimulate IL-1 production by monocytes or macrophages *in vitro*.[2] Our data were similar to their findings, although the dose-responses were slightly different for each glucan.

The adjuvant effect of HD-LE on the antibody response to SRBC was investigated. ICR mice were injected with various samples i.p. and simultaneously with SRBC (1×10^7, i.p). The anti-SRBC PFC responses on day 7 after administration are shown in Table III. The administration of HD-LE 0 h, 0.5 h and 3 h increased the number of PFC immunoglobulin (IgG) to SRBC, although low-molecular-weight HD-LE 6 h did not exhibit such an effect.

As described above, HD-LE possessing a molecular weight of not less than 21000 exhibited significant antitumor activity against sarcoma 180. This result is consistent with other reports concerning the effective molecular weight of antitumor glucans.[7] However, the immunological activities of lower- molecular-weight glucans have not been investigated. Therefore we examined some biological activities, especially macrophage activation, induced by HD-LE of various molecular weights. Since glucan-opsonization with complement has been considered as a possible candidate for the first interaction of glucans and host components, we examined the ability of HD-LE to activate the alternative complement pathway. Glucans with molecular weights of not more than 21000 (HD-LE 3 h) showed reduced activity on the alternative complement pathway (Fig. 1). This finding suggested that HD-LE 3 h was hardly recognized by phagocytes through a third component of complement. Lower- molecular-weight linear (1→3)-β-D-glucan from *Alcaligenes faecalis* var. *myxogenes* IFO 13140 also showed reduced activation of alternative complement pathway.[12] The limit of the degree of polymerization (DP) of glucose residues of the glucan for complement activation was about DP 20, and the effect on the alternative complement pathway of carboxymethylated glucan decreased with increase of the carboxymethyl substitution. These observations suggest the possibility that the critical molecular weight of branched (1→3)-β-D-glucan to activate alternative complement pathway is larger than that of linear glucan.

Next, we examined the effects of HD-LE on macrophage activation *in vitro*. The addition of HD-LE to the culture elevated functions such as glucose consumption, lysosomal enzyme production, and IL-1 production by macrophages. The highest activity on glucose consumption, and on secretion of lysosomal enzyme and IL-1 was observed by stimulation with HD-LE 0 h (MW 800000). These activities decreased with reduction of the molecular weight. Incubation with dextran T-500 which has a high molecular weight of about 500000 showed a lower effect than HD-LE 0 h and 0.5 h. These findings suggested that ultrastructure might correlate to these activities. The adjuvant activity against SRBC of HD-LE was evaluated as a model for antigen-specific responses. HD-LE 0 h, 0.5 h, and 3 h showed augmentative effect on the IgG responses to SRBC, implying that glucan which has a molecular weight of not less than 21000 can stimulate antigen-specific responses, although the response to the administration of HD-LE 3 h was less than that with HD-LE 0 h and 0.5 h.

Thus HD-LE could be classified into three groups by comparing the activities assessed in this paper, namely, 1) HD-LE 0 h (MW 800000) which activates all of the biological activities tested, 2) HD-LE 0.5 h and 3 h (MW 250000 and 21000) which lack or have low activities for activation of the alternative complement pathway and lysosomal enzyme secretion, 3) HD-LE 6 h (MW 6400) which only activate glucose consumption and synthesis of lysosomal enzyme. Examination of physicochemical properties suggested that heat treatment at 150 °C did not alter the primary structure of the parent glucan. However, ultrastructure such as helical conformation of treated glucans was degraded to random coil structure as assessed by using aniline blue (Table I).[13] Therefore, not only the change of molecular weight, but also the conformational change might affect the biological activities. As shown in Table I, the ordered structure of HD-LE 3 h was about 50% of that of GRN LE. This indicated that even half the content of ordered conformation could induce significant biological activities, except for the activation of alternative complement pathway and lysosomal enzyme secretion. High molecular weight and intact ordered structure might be required for the alternative complement pathway and lysosomal enzyme secretion, because even HD-LE 0.5 h which had 82.1% ordered structure showed decreased activities.

This investigation has raised the possibility that antitumor glucans may not always be multiple enhancers of host defense systems, and that high molecular weight and ordered structure are required for extensive enhancement of

immunological activities. The present findings further suggest that heat treatment for 0.5 h to 3 h at 150 °C might be a useful method to solubilize a gel-forming (1→6)-branched (1→3)-β-D-glucan without destroying the antitumor activity.

Acknowledgment We thank Miss S. Fujii for assistance with the preparation of HD-LE and examination of its physicochemical properties.

References

1) N. R. Di Luzio, *Springer Semin. Immunopathol.*, **8**, 387 (1985).
2) J. P. Fruehauf, G. D. Bonnard and R. B. Herberman, *Immunopharmacology*, **5**, 65 (1983); I. Sugawara, K. C. Lee and M. Wong, *Cancer Immunol. Immunother.*, **16**, 137 (1984).
3) H. Wagner, C. Hardt, K. Heeg, K. Pfizenmaier, W. Solbach, R. Bartlett, H. Stockinger and M. Rollinghoff, *Immunological Rev.*, **51**, 215 (1980).
4) J. Hamuro, M. Rollinghoff and W. Hermann, *Cancer Res.*, **38**, 3080 (1976); T. Takeyama, I. Suzuki, N. Ohno, S. Oikawa, K. Sato, M. Ohsawa and T. Yadomae, *J. Pharmacobio-Dyn.*, **10**, 644 (1987).
5) D. W. Dresser and J. M. Phillips, *Immunology*, **27**, 895 (1974); I. Suzuki, K. Hashimoto, S. Oikawa, K. Sato, M. Ohsawa and T. Yadomae, *Chem. Pharm. Bull.*, **37**, 410 (1989).
6) J. Hamuro, U. Hadding and D. Bitter-Suermann, *Immunology*, **34**, 695 (1978); M. Matsushima, U. Sankawa, T. Okuda, H. Okada and T. Sasaki, *Jpn. J. Exp. Med.*, **53**, 219 (1983).
7) T. Sasaki, N. Takasuka, G. Chihara and Y. Maeda, *Gann*, **67**, 191 (1976); T. Sasaki, N. Abiko, Y. Sugino and K. Nitta, *Cancer Res.*, **38**, 379 (1978); T. Kojima, K. Tabata, W. Itoh and T. Yanaki, *Agric. Biol. Chem.*, **50**, 231 (1986); Y. Adachi, N. Ohno, T. Yadomae, Y. Suzuki, K. Sato and S. Oikawa, *Carbohydr. Res.*, **177**, 91 (1988).
8) N. Ohno, Y. Adachi, I. Suzuki, K. Sato, S. Oikawa and T. Yadomae, *Chem. Pharm. Bull.*, **34**, 1709 (1986).
9) A. J. Cunningham and A. Szenberg, *Immunology*, **14**, 599 (1968).
10) H. U. Schorlemmer, D. Bitter-Suermann and A. C. Allison, *Immunology*, **32**, 929 (1977).
11) D. B. Lew, C. C. Leslie, D. W. Riches and P. M. Henson, *Cell. Immunol.*, **100**, 340 (1986).
12) S. Honda, H. Sugino, T. Asano and A. Kakinuma, *Immunopharmacology*, **11**, 29 (1986).
13) P. J. Wood and R. G. Fulcher, *Carbohydr. Polym.*, **4**, 49 (1984).