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FULL PAPER

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Low molecular weight maitake MD-Fraction (Klasma-MD) hydrolyzed with endo-β-1,6-glucanase of *Trichoderma harzianum* induces antitumor activities

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Abstract We previously reported that MD-Fraction (Klasma-MD), a specific-structured β-glucan extracted from fruit bodies of maitake mushrooms (*Grifola frondosa*), can activate cellular immune systems and express antitumor effects. The molecular weight of the MD-Fraction ranged from 1200000 to 2000000. The molecular weight of the products of the MD-Fraction obtained after hydrolysis by endo-β-1,6-glucanase from *Tricoderma harzianum* was reduced to 250000 in terms of dextran, and these products inhibited MM-46 carcinoma in C3H/HeJ mice by 67.9% after intraperitoneal injection. In addition, interleukin-12 and interferon-γ values were increased 1.4 fold and 2.1 fold, respectively, compared with control values. From these results, low molecular weight MD-Fraction (L-MD-Fraction) maintains an immunological active site.

Key words Antitumor action · β-1,6-Glucanases · MD-Fraction · *Tricoderma harzianum*

Introduction

We previously reported that the (1 → 3)-branched (1 →6)-β-glucan termed MD-Fraction extracted from fruit bodies of the maitake mushroom (*Grifola frondosa* (Dicks.) Gray) (Fig. 1), is a biological response modifier (BRM) similar to lentinan extracted from *Letinus edodes* (Berk.) Singer. Orally administered MD-Fraction reduced tumor growth in mice, without affecting body weight (Adachi et al. 1987a,b; Hishida et al. 1988). We postulated that orally administered high molecular weight material activates gut immune systems through intestinal absorption and that low molecular weight MD-Fraction could play a role in this process.

The β-1,6-glucanase lyses yeast in concert with β-1,3-glucanases or proteases, as well as filamentous fungal and bacterial cell walls (Yamamoto et al. 1974; Rombouts et al. 1976; Shibata and Fujimbara 1976; de la Cruz et al. 1995a; de la Cruz and Llobell 1999). *Trichoderma* species produce various hydrolytic enzymes, including chitinases (EC3.2.1.14), β-1,3-glucanases (EC3.2.1.39), β-1,6-glucanases (EC3.2.1.75), and proteases (Elad et al. 1982; de la Cruz et al. 1993, 1995b; Cohen-Kupiec et al. 1999).

Here, we prepared a pure endo-β-1,6-glucanase from *Tricoderma harzianum* T66 (*T. harzianum*) and then examined the antitumor effect of low molecular weight MD-Fraction (L-MD-Fraction).

Materials and methods

Chemicals and reagents

A powder prepared from fruit bodies of maitake mushrooms was obtained from Yukiguni Maitake (Niigata, Japan). Pustulan (isolated from *Umbilicaria papullosa* (Ach.) Nyl.) was purchased from Calbiochem (La Jolla, CA, USA), and laminarin (isolated from *Laminaria digitata* (Hudson) Lamouroux) was purchased from Sigma Chemical (St. Louis, MO, USA). Sephacryl S-200HR, DEAE Sepharose Fast Flow, and CM Sepharose Fast Flow were purchased from Pharmacia Biotech (Uppsala, Sweden). Shodex OHpak SB-2006M was supplied by Showa Denko (Tokyo, Japan). Ultrafiltration membranes (NMWL 5000) were obtained from Millipore (Billerica, MA, USA).

Organisms and growth conditions

Trichoderma harzianum T66 (ATCC MYA-1175) obtained from the American Type Culture Collection (Manassas, VA, USA) was maintained on malt extract agar. Conidia

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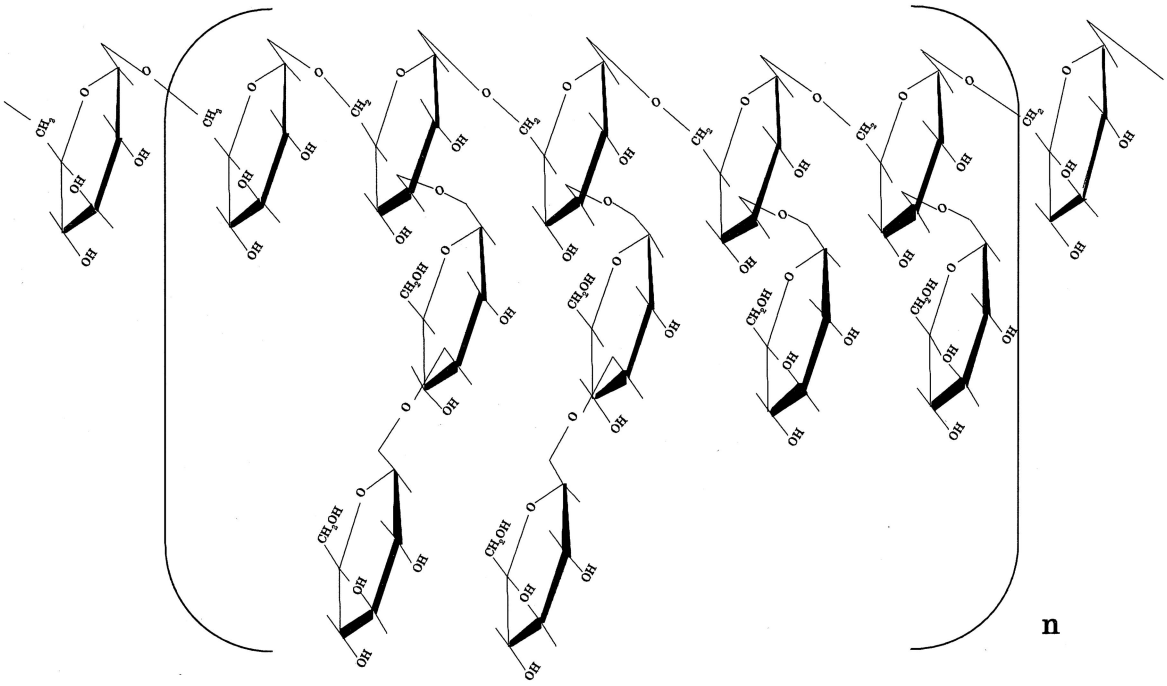

Fig. 1. Chemical structure of MD-Fraction (Klasma-MD)

inoculated in Waksman medium [0.1% (w/v) potassium phosphate, 0.05% (w/v) magnesium sulfate, 1.0% (w/v) glucose, 0.5% (w/v) peptone, 0.8% (v/v) phosphoric acid, pH 3.8] were rotary incubated for 14 days at 27°C at 110 rpm.

Enzyme and protein assays

The activity of β-1,6-glucanase was determined by measuring the amount of reducing saccharide released from pustulan. The standard assay (1 ml) was a mixture containing 0.5 ml 1.0% (w/v) pustulan in 50 mM acetate buffer (pH 5.0) and 0.5 ml enzyme appropriately diluted in the same buffer. The reaction mixture was incubated at 45°C for 30 min and boiled for 10 min to stop the reaction. The reducing saccharide content was determined by the procedure of Somogyi and Nelson (Somogyi 1952; Nelson 1955). Standards for glucose, as well as enzyme and substrate blanks, were also incubated. The substrate was replaced by laminarin in the β-1,3-glucanase assay. One enzymatic unit was defined as the amount of enzyme required to catalyze the release of reducing saccharide groups equivalent to 1 μmol glucose min^{-1} under the above assay conditions. Protein concentration was measured by the Lowry method, using bovine serum albumin as the standard (Lowry et al. 1951).

Preparation of MD-Fraction

Powdered MD-Fraction was prepared from fruit bodies of maitake mushrooms as described (Kodama et al. 2001). Polysaccharide concentration was determined by the anthrone method (Dreywood 1946), and protein was detected using the Lowry method.

Enzyme purification

Unless otherwise indicated, all steps proceeded at temperatures ranging from below 0 to 4°C. *Trichoderma harzianum* cultured for 14 days in Waksman medium was filtered, and the filtrate was separated by passage through a Sephacryl S-200 HR column (3.0 × 50 cm) equilibrated in 50 mM sodium acetate buffer, pH 5.0, at a flow rate of 2.0 ml/min. Fractions were assayed for β-1,6-glucanase activity, and active fractions were concentrated by ultrafiltration.

Concentrates were applied to a DEAE Sepharose Fast Flow column (3.2 × 30 cm) equilibrated with 50 mM sodium acetate buffer, pH 5.0. Proteins were eluted using a 0–0.2 M NaCl gradient in the same buffer at a flow rate of 0.66 ml/min. The β-1,6-glucanase activities of each fraction were detected as peaks I and II. The active fractions were pooled and dialyzed with 50 mM sodium acetate buffer, pH 5.0, and concentrated by ultrafiltration. Peak I was again applied to a DEAE Sepharose Fast Flow column (2.5 × 40 cm) equilibrated with the same buffer, and the enzyme was eluted using a 0–0.1 M NaCl gradient in the same buffer. Peak II was eluted using a 0.1–0.2 M NaCl gradient in the same buffer. The most active fractions of each peak were collected, concentrated by ultrafiltration, and dialyzed with 50 mM sodium acetate buffer, pH 5.0.

The molecular weight of β-1,6-glucanase of peak I was estimated by sodium dodecyl sulfate-polyacrylamide gel

Table 1. Preparation of β-1,6-glucanases of *Trichoderma harzianum*

	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Culture medium	3150	22.27	1225.0	0.018	100.0	1.00
Sephacryl S-200 HR	6300	24.50	1176.9	0.021	110.0	1.15
DEAE Sepharose FF (1)						
Peak I	2.4	8.04	8.57	0.938	36.1	51.6
Peak II	3.0	14.85	17.64	0.842	66.7	46.3
DEAE Sepharose FF (2)						
Peak I	4.0	6.48	3.68	1.761	29.1	96.9
Peak II	4.0	11.64	6.76	1.722	52.3	94.7

electrophoresis (SDS-PAGE) in 15% acrylamide gel containing 0.1% SDS. Myosin (MW = 199000), β-galactosidase (MW = 131000), soybean trypsin inhibitor (MW = 30800), and lysozyme (MW = 17400) were used as standards. Gel was stained with Silver Stain MS Kit (Wako Pure Chemical Industries, Osaka, Japan).

Analysis of β-1,6-glucanase reaction products

Pustulan 0.5% (w/v) was reacted with 0.0135 U/ml β-1,6-glucanase in 50 mM sodium acetate buffer, pH 5.0, for periods ranging from 30 min to 4 h. The products were analyzed by high-performance liquid chromatography (HPLC) on a Shodex OHpak SB-2006M column (20 × 300 mm) maintained at room temperature and equilibrated with 5 mM phosphate buffer, pH 7.0. The same buffer was the elution solvent at a flow rate of 3.0 ml/min. Hydrolyzed products were detected by the anthrone method and identified by comparison with glucose or dextran standards.

Preparation of L-MD-Fraction

MD-Fraction (6.0 mg/ml) was mixed with 0.12 U/ml β-1,6-glucanase (peak I) in 50 mM sodium acetate buffer, pH 5.0, and incubated at 45°C for 4 h. The L-MD-Fraction was fractionated using a Shodex OHpak SB-2006M column (20 × 300 mm).

Antitumor activity of MD- and L-MD-Fractions

MM-46 carcinoma cells (1×10^6) were implanted in the right axillary region of 7-week-old male C3H/HeJ mice at Japan Clea (Osaka, Japan). After 24 h, either MD- or L-MD-Fraction (4 mg/kg per day each) was injected i.p. for 19 consecutive days into the tumor-bearing mice. Control mice received phosphate-buffered saline (PBS (–)).

Measurement of interleukin (IL)-12 and interferon (IFN)-γ production

Levels of secreted IL-12 and IFN-γ were determined by enzyme-liked-immunosorbent assay (ELISA) in mouse blood obtained at days 0, 5, 10, and 20 and then stored at –85°C. Purified antimouse IL-12 (p40/70) was purchased from Biolegend (San Diego, CA, USA). Recombinant murine IL-12, biotinylated antimurine IL-12, biotinylated antimurine IFN-γ, and recombinant murine IFN-γ were purchased from PeproTech EC (London, UK). Rat antimouse IFN-γ was purchased from PBL Biomedical Laboratories (Piscataway, NJ, USA).

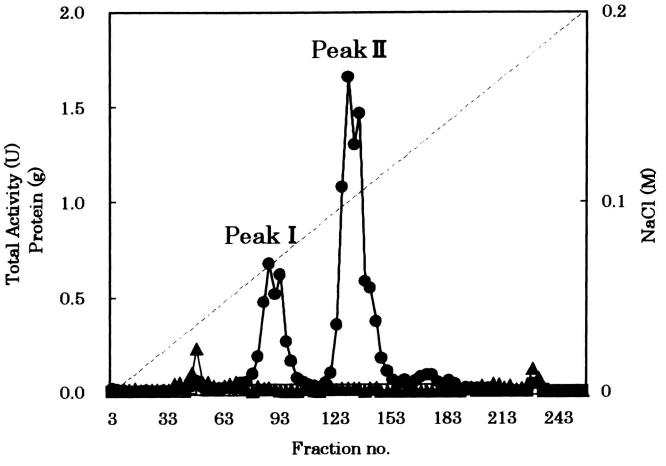

Fig. 2. Enzyme preparations from anion-exchange column chromatography. Column, DEAE Sepharose Fast Flow (ϕ 3.2 × 30 cm); buffer, 50 mM NaOAc (pH 5.0); elution buffer, 0–0.2 M NaCl in 50 mM NaOAc (pH 5.0); flow rate, 0.66 ml/min; fractions, 16 ml. ●, β-1,6-glucanase activities; ▲, β-1,3-glucanase activities; □, protein

Results

Enzyme production and preparation

Preparations of β-1,6-glucanases were separated by Sephacryl S-200HR gel filtration, and then two peaks of β-1,6-glucanase were separated by DEAE Sepharose Fast Flow (Fig. 2). Peaks I and II were eluted at 0.07 M and at 0.15 M NaCl, respectively, in 50 mM NaOAc (pH 5.0). The overall purification was 97 fold with a recovery of 30% for peak I and 95 fold with a recovery of 52% for peak II (Table 1). The molecular weight of peak I was estimated by SDS-PAGE to be 18 kDa (Fig. 3).

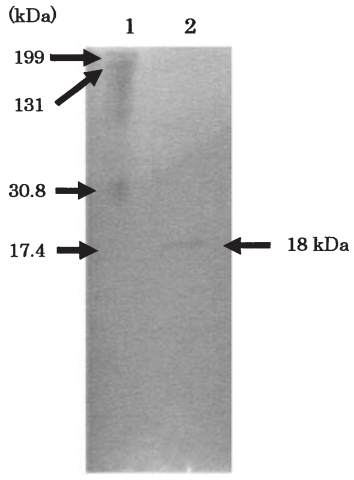

Fig. 3. SDS-PAGE for purification of enzyme β-1,6-glucanase of peak I. The gel was stained with a Silver stain MS kit. *Lane 1*, standard protein markers; *lane 2*, β-1,6-glucanase of peak I eluted from DEAE Sepharose Fast Flow column

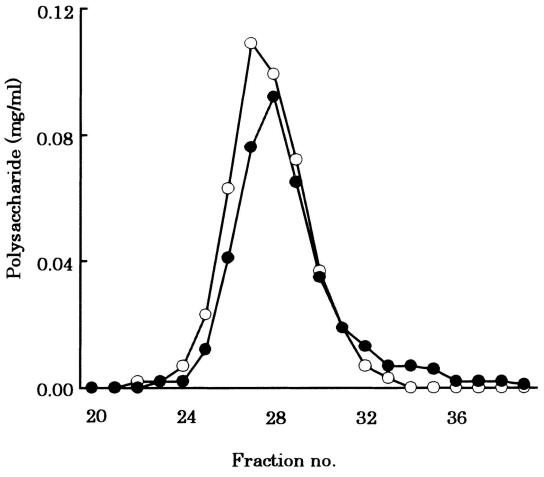

Fig. 4. HPLC analysis of β-1,6-glucanase of peak I action on MD-Fraction. Column, Shodex OHpak SB-2006M (20 × 300 mm); buffer, 5 mM phosphate (pH 7.2); flow rate, 3.0 ml/min; fractions, 1.5 ml; injection, 1.0 ml containing 1.0 mg/ml. ●, MD-Fraction was reacted with β-1,6-glucanase of peak I; ○, MD-Fraction

Substrate specificity and activity

Peaks I and II appeared to be specific β-1,6-glucanases, but the ratio of β-1,6- and β-1,3-glucanase in each peak was 99.2:0.8 and 98.2:1.8, respectively.

To determine whether the β-1,6-glucanases of peaks I and II were endo- or exoglucanases, we analyzed the products released via the enzymatic hydrolysis of pustulan by HPLC. The molecular weight of the released product was 17500 and 10000 when pustulan was digested with either of the peaks I or II β-1,6-glucanases for 4 and 6 h, respectively. Glucose release was undetectable in the hydrolysis products of either enzyme, suggesting that the β-1,6-glucanases of peaks I and II are endoglucanases.

Preparation of L-MD-Fraction

The MD-Fraction was incubated with β-1,6-glucanase (peak I), and the material released was analyzed by HPLC. The products from MD-Fraction generated by the peak I enzyme appeared at a retention time (RT) of about 16.0 min (Fig. 4). Fractions at 15.5–19.5 min of RT were collected, evaporated by aspiration, and the concentrate was defined as L-MD-Fraction. The molecular weight of L-MD-Fraction was 250000 as determined by HPLC (Fig. 5).

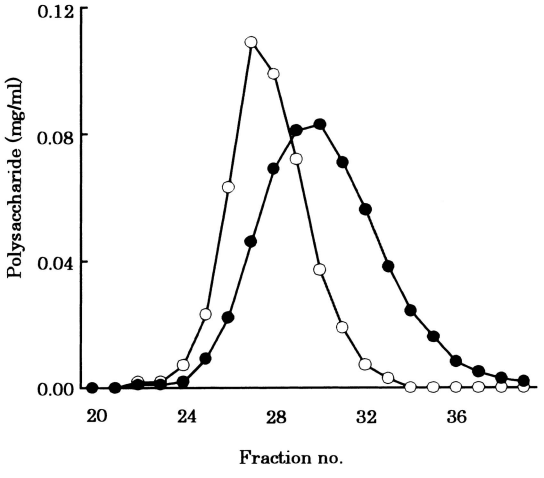

Fig. 5. HPLC analysis of MD- and L-MD-Fractions. Column, Shodex OHpak SB-2006M (20 × 300 mm); buffer, 5 mM phosphate (pH 7.2); flow rate, 3.0 ml/min; fractions, 1.5 ml; injection, 1.0 ml containing 1.0 mg/ml. Molecular weight determined from dextran conversion. Glucose elution detected at RT 23.0 min. ●, L-MD-Fraction; ○, MD-Fraction

Antitumor activity of L-MD-Fraction

The tumor inhibition ratio (TIR) of the L-MD- and MD-Fractions as a ratio (%) of the PBS (–) control on day 20 were 67.9% and 54.6%, respectively (Fig. 6).

The amount of IL-12 in plasma from mice treated with the L-MD-Fraction increased on day 20, whereas the maximal amount produced by mice treated with the MD-Fraction and PBS (–) was decreased on day 5. The production of IFN-γ peaked in all samples, on day 5, at the start of which the amount of IFN-γ was decreased (Fig. 7).

Discussion

We confirmed that the β-1,6-glucanases of *T. harzianum* found in peaks I and II were endoglucanases (see Fig. 2). The β-1,6-glucanase of peak I hydrolyzed the β-1,6-bonds in the main chain of the MD-Fraction. The molecular weight of fractionated L-MD-Fraction was 250000, which was one-sixth that of the MD-Fraction. The MD-Fraction was less susceptible to hydrolysis with β-1,6-glucanase than pustulan because of its high molecular weight glucan, having β-1,6-bonds in the main chain and β-1,3-bonds in side chains.

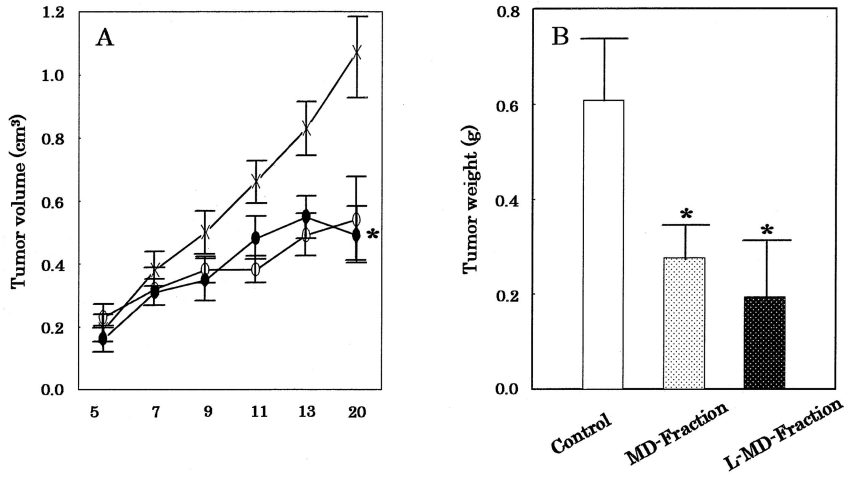

Fig. 6. Effect of MD- and L-MD-Fractions on MM46 carcinoma cell growth. MD-Fraction (4 mg/kg per day) was administered i.p. to MM-46 carcinoma-bearing mice for 19 consecutive days. Tumor volume (cm^3 = longest × shortest $cm^2/2$) (**A**) and weight (**B**) were determined on day 20. Data are expressed as means ± SEM of five experiments. $^*P < 0.05$ compared with control mice given saline (Student's *t* test). ●, L-MD-Fraction; ○, MD-Fraction; ×, saline

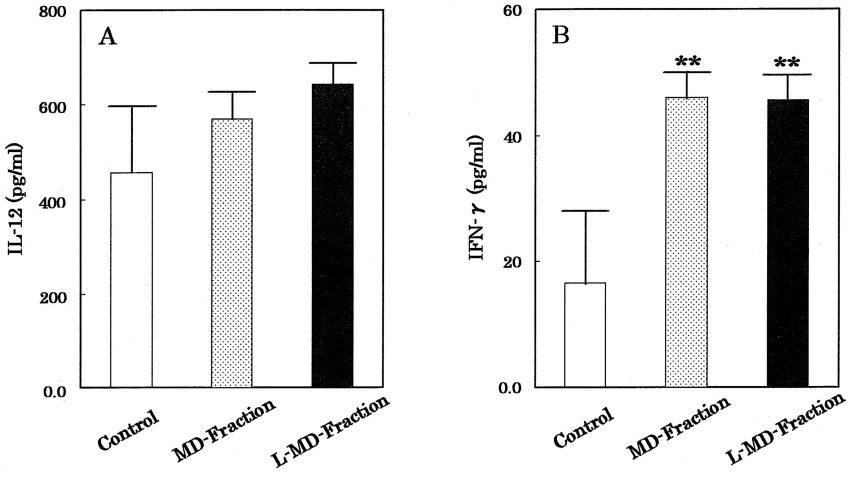

Fig. 7. Production of interleukin (IL-12) and interferon (IFN)-γ in plasma elicited by MD- and L-MD-Fractions on day 20. Production of IL-12 (**A**) and IFN-γ (**B**) in plasma was measured using enzyme-linked immunosorbent assay (ELISA). Data are expressed as means ± SEM of five experiments. $^{**}P < 0.01$ compared with control mice given saline (Student's *t* test)

L-MD- or MD-Fractions significantly decreased the growth of MM-46 carcinoma in mice compared with PBS (–). The L-MD-Fraction inhibited growth of the MM-46 carcinoma on day 20 to 0.49 ± 0.05 cm^2 compared with 1.07 ± 0.22 cm^2 in mice treated with PBS (–) ($^*P < 0.05$; see Fig. 6). To clarify this regression, we measured IL-12 production. Levels of IL-12 were maximal at day 20 (1.4 fold higher than those in mice administered with saline) among tumor-bearing mice administered with the L-MD-Fraction. On the other hand, the production of IFN-γ was 2.1 fold that of mice injected with saline at day 20 ($^{**}P < 0.01$ compared with control mice; see Fig. 7). We considered that immunological activation was continuously increased more by the L-MD-Fraction than by the MD-Fraction.

$CD4^+$ T cells are classified into three subtypes based on cytokine production as follows: (1) helper (Th)-1 cells, which produce IL-2, IFN-γ, and tumor necrosis factor (TNF)-α and introduce cellular immunity to the organism; (2) Th-2 cells, which produce IL-4, IL-5, IL-6, IL-10, and Il-13 and activate humoral immunity; and (3) precursor or Th-0 cells. Cellular immunity is also induced by IL-1β, IL-12, and IL-18 produced by antigen-presenting cells (APC) (Inoue et al. 2002). We previously described that the MD-Fraction induced a Th-1-dominant response in C3H/HeN mice bearing MM-46 carcinoma, indicating an antitumor effect (Inoue et al. 2002; Kodama et al. 2001). The L-MD-Fraction similarly induced a Th-1-dominant response in these mice. We considered that the L-MD-Fraction induced cellular immunity enhanced by the production of IL-12 by APC and IFN-γ by Th-1 cells.

The results of the present study indicated that the low molecular weight component of the MD-Fraction is an immunomodulator, and that the antitumor effect is structure specific and not associated with the molecular weight. The antitumor activity of MD-Fraction is decreased when β-1,3 bonds of side chains in MD-Fraction were cut by β-1,3-glucanase (data not shown). Therefore, structural analysis of potentially therapeutic compounds is essential for clarification of the immune system, including the mechanisms by which the oral administration of high and low molecular weight MD-Fractions conferred immunity through intestinal absorption.

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