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INFLUENCE OF STRUCTURAL FEATURES ON IMMUNOSTIMULATING ACTIVITY OF GLUCANS EXTRACTED FROM *AGARICUS BLAZEI* MUSHROOM

ABSTRACT: High molecular weight β-D-glucans derived from *Basidiomycetes* cell walls are able to specifically activate cellular and humoral components of the host immune system. The aim of this paper was to examine immunomodulating activity of native, chemically and enzimatically modified glucans from *Agaricus blazei* mushroom and to determine which structural features are of primary importance for their stimulation referring to humane immune cells. The immunomodulating activities were tested *in vitro*, by stimulation of peripheral blood mononuclear cells (PBMCs) and measuring of interferon-gamma (IFN-γ) production by enzyme linked immunosorbent assay (ELISA). Measurements of immunomodulatory capacity of *Agaricus blazei* native glucans showed their expressive immunostimulating effect on activated PBMCs and synthesis of IFN-g. The results obtained after the stimulation of cells with 1M H_2SO_4 and 1M NaOH, the treated glucans showed that primary structure is of more importance than the tertiary structure of the triple helix for their immunostimulating activity and synthesis of IFN-γ. Glucans of lower molecular weight obtained after acid hydrolysis appeared as effective immunostimulators of PBMC's. The results obtained after the incubation of cells with 1,6 β-glucanase modified glucans suggest that β-(1,6) binding of glucose monomers probably has no importance for the production of imunostimulating effects, *in vitro*. This confirmed that β-(1,3) bonds are the primary determinants of immunomodulatory activities and stimulation of IFN-γ synthesis.

KEY WORDS: *Agaricus blazei*, β-D-glucans, IFN-γ, immunostimulating activity, PBMC's

INTRODUCTION

β-D-glucans are important secondary metabolites isolated from microorganisms, mushrooms and plants. They exhibit prophylactic and therapeutic properties and can function as biological response modifiers when administered to mammals. They have the ability to enhance or suppress both innate and

acquired immune response. The major immunopotentiation effects of these active substances include mitogenicity and stimulation of hematopoietic stem cells, such as T_H and T_C cells, B cells, macrophages, DC_s, and NK cells (Lull et al., 2005.).

The term "β-glucan" refers to the polymers which are generally composed of a linear backbone containing D-glucopyranosyl repeat units which are linked together by β-(1,3) and β-(1,4)-linkages. Some, but not all, exhibit β-(1,6)-side chains on the backbone. Glucans can assume a number of solution conformations depending upon the solvent system. For water soluble glucans, the two predominant conformations are single helix or triple stranded right--winding helix. In the fungal cell wall, most glucans comprise a three-dimensional network of β-(1,3-1,6)-glucans that are connected to the other carbohydrates, proteins and lipids (Young and Castranova, 2005).

The mechanism of the imunomodulating effect of glucans is not yet fully understood and probably depends on chemical characterisics, such as molecular weight, branching patterns, solubility in water and conformational features like the formation of helix (Freimund et al., 2003).

In this study we tried to determine which structural features of water soluble glucans are of primary importance for their *in vitro* immunostimulatory properties. Two types of glucan structure modification were applied, a chemical with 1M NaOH and 1M H_2SO_4 and enzymatic with 1,6 β-glucanase. NaOH changes the conformation of glucans from triple helix to single strand (Maeda et al., 1988). Glucans of lower molecular weight were obtained after acid hydrolysis with 1M H_2SO_4 (Dia et al., 2003) and modification with 1,6 β-glucanase.

MATERIAL AND METHODS

Glucans were extracted by hot water and alcohol precipitation from powder of fruit body of *Agaricus blazei* mushroom. Purification of extract was done by dialysis. The immunomodulating activity of native and modified glucans was tested *in vitro*, by PBMCs and measurement of IFN-g production was done by ELISA. Changes of molecular weight, after incubation with 1M NaOH, 1M H_2SO_4 and 1,6 β-glucanase, were observed by exclusion chromatography using Sephacryl S 200 (Kozarski, 2006.).

Extraction of water soluble glucan fraction

Up to 10% of dried powdered tissue was suspended in water. Glucans extraction was done by autoclaving 2 x at 121°C for 20 minutes. The extract was cooled down and centrifuged at 12325 x g for 20 minutes. Supernatants were collected and boiled to 10% of starting volume. Two volumes of 96% ethanol were added and left at 4°C overnight. Supernatant was decanted, washed 1 x with 70% ethanol and centrifuged at 12325 x g. Pellet was dried at 42°C. Purification was done by dialysis, against 2 l of destilled water, obtained by Millipore purification system (MilliQ) for 24 hours at room temperature.

Chemical modification

50 mg/ml of glucans were incubated in 1M NaOH and 1M H_2SO_4 at 37°C for 16 hours. Neutralization was done with 10M H_2SO_4 resp. 10M NaOH to pH 6.8—7.2. Glucans in 1M phosphat buffer saline (PBS) were used as control, under the same conditions.

Enzymatic modification

25 mg/ml of glucans in 5mM sodium acetate (NaAc) buffer at pH 5.4 were incubated with 6 mg/ml of 1,6 β-glucanase, Onuzuka R-10 (Yakult Honsha Co Ltd., Japan) for 1 hour at 55°C. Reaction was stopped by heating at 90°C for 30 minutes followed by precipitation of glucans by addition of 2 volumes of 96% ethanol.

Column chromatography

Size exclusion chromatography was done on a 1.5 x 90 cm column of Sephacryl S 200. 25 mg/ml of each glucan sample in MilliQ applied on column. Eluation was done using fast performance liquid chromatography (FPLC) system (Pharmacia) with degassed MilliQ water at flow rate of 0.5 ml/min. The eluents were collected by a fraction collector (Pharmacia), each 5 ml in a tube. The void volume was determined to be 60 ml in each fraction, and glucan content was semiquantified by the phenol-sulfuric acid method with glucose as a reference (Dubois et al., 1956). Protein content was determined using the Bradford method with bovine serum albumun (BSA) as a standard (Bradford et al., 1976) Glucan and protein contents were measured in 5 times concentrated fractions.

Cells isolation

Human PBMCs were prepared from buffy coats, obtained from various healthy donors. Buffy coats were diluted 1 x with PBS and centrifuged for 15 minutes at 2500 x g over a layer of Histopaque 1077 (Sigma). PBMCs were carefully collected at the interphase and washed with PBS. Cells were counted and resuspended at 5—10 x 10^6/ml in RPMI-1640 containing 10% fetal calf serum (FCS), 1% penicilin and 1% streptomycin.

Immunomodulatig activity

Glucan solutions were heated before application at 95°C for 20 minutes. Immunomodulating activity of the various glucans was tested by exposing stimulated PBMCs. As transcription activators, 1 ng/ml phorbol 12-myristate

13-acetate (PMA) and 0,5 µl/ml Ca-ionophore were added. After incubation for 48 hours at 37°C in 5% CO_2 atmosphere, medium was tested for IFN-g concentration by sandwich ELISA.

ELISA was performed in Nunc Maxisorp high affinity 96 well plates. Wells were coated overnight at 4°C wih 50 µl of mouse anti-human IFN-γ in PBS, pH 7. Blocking of non specific binding sites was carried out overnight at 4°C with 0,1% BSA and 1% skimmed milk protein in PBS. Plate was incubated for 2 hours at room temperature with samples diluted in reagent diluent (0.1% BSA, 0.05% Tween 20 in Tris buffered saline, pH 7.2—7.4, 0.2 µm filtered) and duplicates of standard, human IFN-g (BioSource) reconstituted with 50% glycerol, that was serially diluted in reagent diluent. The concentration of high standard was 1000 pg/ml. The 100 µl biotinylated goat anti-human IFN-g in reagent diluent was added to each well, followed by 1 hour incubation at room temperature. Streptavidin-HRP (BioSource) in 100 µl of reagent diluent was added to the wells and incubated for 20 minutes at room temperature. After each step, the plate was washed with wash buffer (0.05% Tween 20 in PBS, pH 7.2—7.4). Then 100 µl of substrate solution (1:1 mixture of H_2O_2 and tetramethylbenzidine) was added to each well and incubated at room temperature. After 20 minutes, 50 µl of stop solution (1M H_2SO_4) was added. IFN-g production was measured at 415 nm using Benchmark microplate spectrophotometer (Bio-Rad).

RESULTS AND DISCUSSION

Size exclusion chromatography of *A. blazei* native glucans on Sephacryl S 200 showed the presence of one highest peak eluting together with the void volume of the column, indicating high molecular weight, over 80 kDa, and a few small peaks containing molecules of much lower size (Figure 1). In each fraction, the presence of protein was confirmed. This suggested that glucans can bound to protein or peptide residues and form proteoglucans. Running the glucanase digested glucans on the column showed a high fractionation of the glucan extract (Figure 1). This confirmed that glucans of *A. blazei* fruiting bodies predominantly had a β-(1,6)-backbone structure with β-(1,3)-side branches (K o z a r s k i, 2006).

Measurements of immunomodulatory capacity of *Agaricus blazei* native glucans showed that *A. blazei* glucans express immunostimulating effect on activated PBMCs and synthesis of IFN-g (Figure 2). Titers of IFN-g measured after stimulation of cells with acid-hydrolyzed fractions confirmed that glucans of lower molecular weight are as effective as non-hydrolyzed glucans. ELISA measurements of IFN-g titer obtained after the stimulation of PBMCs with 1M NaOH treated glucan showed that the immunostimulating activity was not changed (Table 1, Figure 2). This indicated that the primary structure of glucans is of more importance than the tertiary structure of the triple helix for their immunostimulating activity and synthesis of IFN-γ.

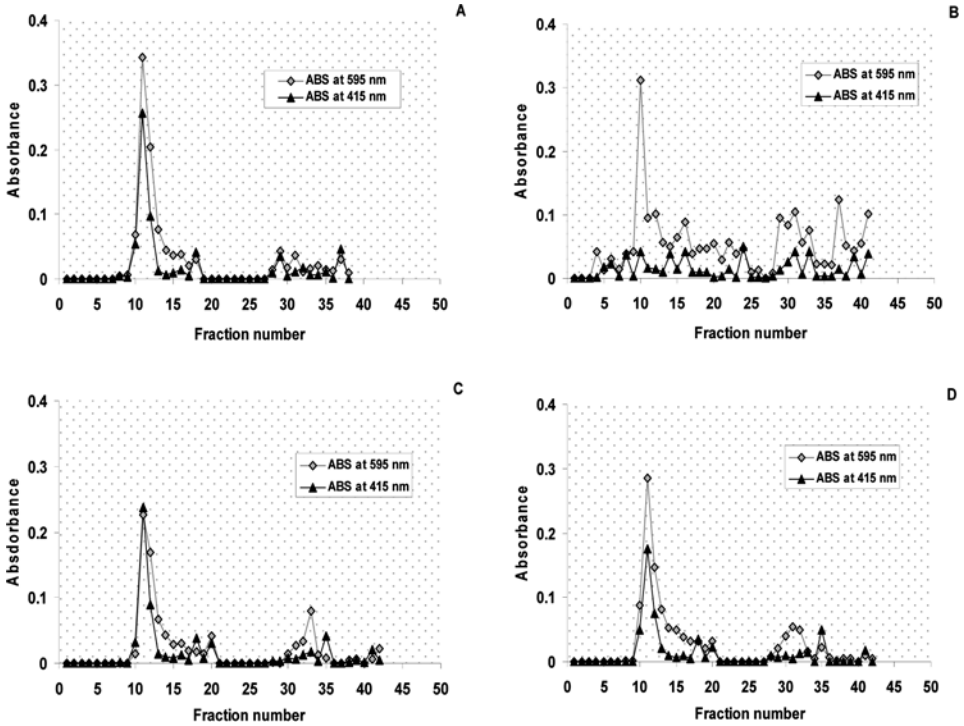

Fig. 1 — Separation of native and modified *A. blazei* (graphs **A**, **B**, **C, D**) fruiting body glucans on Sephacryl S 200. Glucans in PBS, **A**; with 1,6 b-glucanase modified glucans **B**; with 1M H_2SO_4 treated, **C**; and with 1M NaOH, **D**.

Tab. 1 — ELISA measurements of IFN-g titer obtained after the stimulation of PBMCs with *A. blazei* native, in 1 M PBS glucans (**Ab**) and 1M NaOH (**AbB**) and 1M H_2SO_4 treated glucans (**AbA**). Cell suspension in RPMI, with transcription activators, was used as control.

sample	*IFN-γ titer (pg/mL)*
Control	**56.09 ± 4.11**
Ab	**219.26 ± 29.45**
AbB	**215.95 ± 23.72**
AbA	**198.31 ± 25.09**

The resulting β-(1,3)-glucan fragments of high molecular weight (MW > 80 kDa) and small β-(1,3)-glucan fragments (MW < 80 kDa), left after glucanase degradation, showed a strong enhancement of immunostimulatory activity compared to the native glucans (Figure 3).

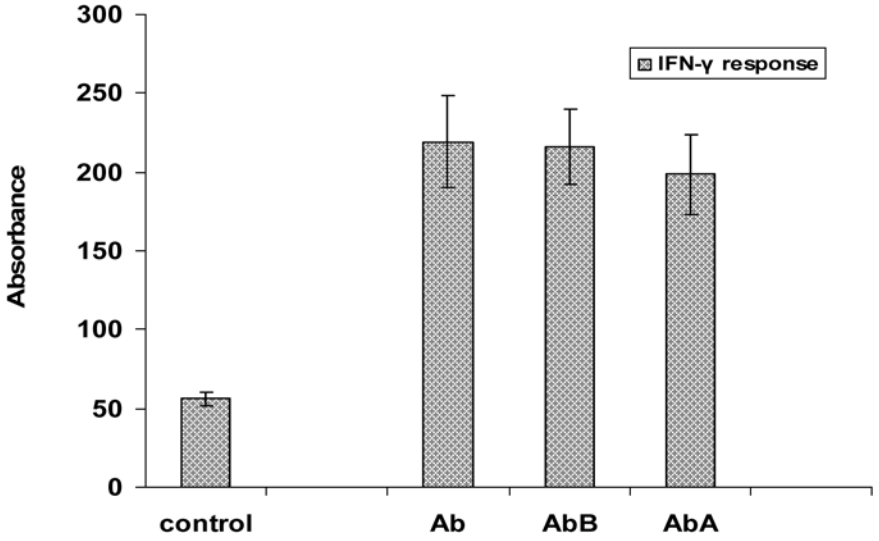

Fig. 2 — IFN-g response of stimulated PBMC's incubated with native, in 1M PBS, *A. blazei* glucans (**Ab**) and glucans which have been exposed to 1M NaOH (**AbB**) and 1M H_2SO_4 (**AbA**).

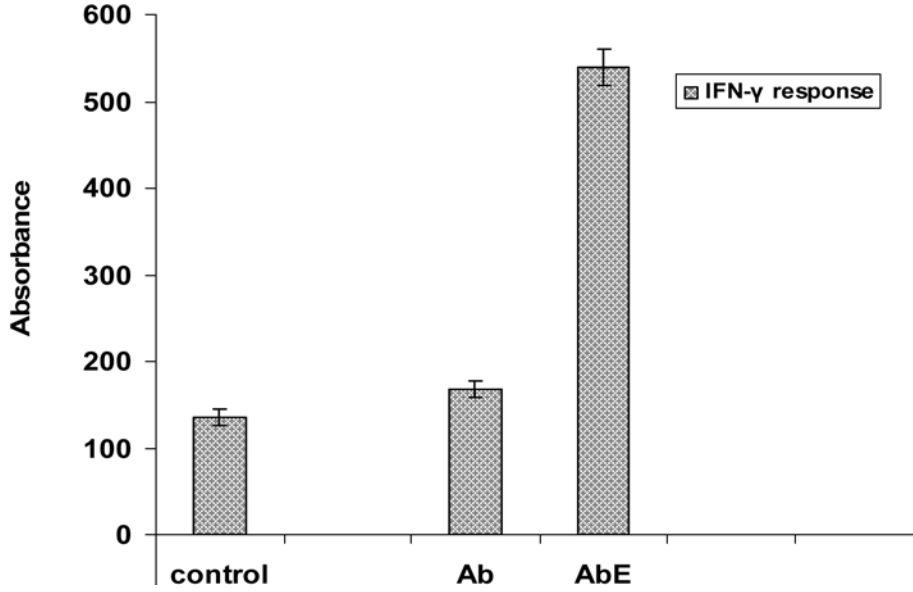

Fig. 3 — IFN-g response, after 48 hours, of stimulated PBMC's incubated with native, in 1M PBS, *A. blazei* glucans (**Ab**) and with 1,6 b-glucanase digested glucans (**AbE**).

Tab. 2 — ELISA measurements of IFN-g titer obtained after the stimulation of PBMCs with *A. blazei* native, in 1 M PBS glucans (**Ab**) and with 1,6 b-glucanase treated glucans (**AbE**). Cell suspension in RPMI, with transcription activators, was used as control.

sample	*IFN-γ titer (pg/mL)*
Control	**135.23 ± 9.30**
Ab	**219.26 ± 29.45**
AbE	**198.31 ± 25.09**

The obtained results suggest that β-(1,6) binding of glucose monomers probably has no importance for the production of imunostimulating effects, *in vitro*.

CONCLUSION

Measurements of immunomodulatory capacity of *Agaricus blazei* native glucans showed that *A. blazei* glucans express immunostimulating effect on the activated PBMCs and synthesis of IFN-g. The results obtained after the stimulation of cells with chemical and enzimatically modified glucans showed that primary structure is of more importance than the tertiary structure of the triple helix for their immunostimulating activity and synthesis of IFN-g. The obtained results confirmed that β-(1,6) binding of glucose monomers probably has no importance for the production of imunostimulating effects, *in vitro*. This suggests that β-(1,3) bonds are the primary determinants of immunomodulatory activities and stimulation of IFN-γ synthesis. The results confirmed that glucans of lower molecular weight are effective for stimulation of PBMCs and production of IFN-γ.

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УТИЦАЈ СТРУКТУРНИХ КАРАКТЕРИСТИКА НА ИМУНОСТИМУЛАТИВНУ АКТИВНОСТ ГЛУКАНА ЕКСТРАХОВАНИХ ИЗ ГЉИВЕ *AGARICUS BLAZEI*

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Резиме

β-D-глукани великих молекулских маса изоловани из ћелијског зида гљива из класе *Basidiomycetes* имају способност да специфично активирају целуларне и хуморалне компоненте имуног система домаћина. Имуномодулаторска активност мико-D-глукана у функцији је њихових хемијских карактеристика, као што су молекулска маса, степен гранања, растворљивост у води и терцијарна структура. Циљ овог рада је био испитивање имуномодулаторске активности нативних, хемијски и ензимски модификованих глукана гљиве *Agaricus blazei* и да се утврди која је структурна карактеристика од примарног значаја за стимулацију ћелија хуманог имуног система. Имуномодулаторска активност је тестирана *in vitro*, стимулацијом мононуклеарних ћелија крви из периферног крвотока (PBMC) молекулима глукана и мерењем количине синтетисаног интерферона-гама (IFN-γ) од стране стимулисаних ћелија ензимоимунотестом (ELISA). Мерењем имуномодулаторског капацитета *Agaricus blazei* нативних глукана показано је да ови молекули имају изражено имуностимулативно дејство на активиране PBMC ћелије и стимулацију синтезе IFN-γ. Стимулацијом ћелија глуканима који су претходно били парцијално хидролизовани 1M H_2SO_4 и 1M NaOH измерени титар IFN-γ се није значајно променио у односу на нативне молекуле. Глукани мањих молекулских маса, настали након киселе хидролизе, показали су се као ефикасни стимулатори PBMC ћелија. Мерењем титра IFN-γ насталог након инкубације активираних ћелија са 1,6 β-глуканазама модификованим глуканима потврђено је да су фрагменти β-(1,3)-глукана великих молекулских маса (ММ > 80 kDa) и мали фрагменти β-(1,3)-глукана (ММ <80 kDa), настали након ензимске модификације, испољили значајно повећање имуностимулативне активности у односу на нативне молекуле. Добијени резултати су указали да β-(1,6)-гликозидне везе немају значаја у испољавању имуностимулативног ефекта, *in vitro*.

Овим је потврђено да је за имуностимулативну активност и стимулацију синтезе IFN-γ од примарног значаја присуство β-(1,3)-гликозидних веза. Закључено је да је за имуномодулаторску активност ових молекула битна примарна структура, а не конформација троструког хеликса нативних молекула, као и да су молекули глукана мањих молекулских маса ефикасни стимулатори синтезе IFN-γ.