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Saccharomyces uvarum Mannoproteins Stimulate a Humoral Immune Response in Mice

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ABSTRACT

Yeasts discarded in industrial processes can be used as a nutritional supplement and to extract cellular components with biotechnological aims. In this study, the humoral immune response of Swiss mice treated with mannoproteins (MP) from the yeast Saccharomyces uvarum *was evaluated. The mice were treated with MPs at different doses and times and inoculated with 2% sheep red blood cells. An increase in total Ig in mice treated with 100 μg of MP at the time of immunization or 24 h before was observed in the primary immune response; in the secondary immune response, an increase was observed in total Ig values for all groups, and an increase of IgG was observed in the mice treated with MPs (100 μg) at the time of immunization or 24 h before. These results show that* S. uvarum *MPs present an immunostimulatory action on the humoral immune response in mice.*

Key words: glycoprotein; Swiss mice; antibody production; yeast; cell wall

INTRODUCTION

Yeast cells are widely used in baking and in the production of wine, beer and spirits (Yamada and Sgarbieri 2005). In Brazil, yeast production is intended mainly for baking, brewing and the distillation of alcohol from sugar. The yeast acts as an agent of biological transformation, and at the end of the production process, large volumes of cells are generated and often discarded as a byproduct (Costa 2008). Thus, it is important to search for other applications for these microorganisms to reduce the environmental impact caused by disposal (Costa 2008).

Yeast, especially from the genus *Saccharomyces*, is gaining more attention as a nutritional supplement and as a flavoring in foods due to its high levels of protein and B vitamins (Sgarbieri et al. 1999). They can be used directly, when intact, or be processed to obtain derivatives, such as autolysate, yeast extract and protein concentrates (Vilela et al. 2000a; Yamada et al. 2003). Currently, there is a tendency to use yeast by isolating some of its major cellular constituents, such as enzymes, proteins, polysaccharides and lipids, which, when isolated, have interesting technological properties and high added values (Vilela et al. 2000b; Fukuda et al. 2009).

Mannoproteins are glycoproteins in the cell walls of yeast with enzymatic or structural functions. Structural mannoproteins are the most abundant. They have a small protein portion covalently linked to a greater fraction of carbohydrates, interspersed throughout a network of glucan to

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form the outer cell wall (Cid et al. 1995; Lukondeh et al. 2003). They are composed of a central protein chain, in which two types of mannan chains are linked, a long bulky central chain (40-100 units) consisting of mannopyranosyl with α-1,6 links, modified along its length by numerous branches of residues of phosphorylated α-1,2- and α-1,3-mannopyranosyl, and the other formed by a short mannose chain of 1-5 units (Lehle 1980; Lesage and Bussey 2006). Mannoproteins with enzymatic functions contain a greater fraction of protein in their compositions and are mainly located in the periplasmic space between the plasma membrane and cell wall (Cid et al., 1995; Lukondeh et al. 2003).

Mannoproteins in the cell walls of yeasts have the ability to stimulate host immunity, interacting with different cells and proteins in the immune system (Casanova 1992). Several studies have demonstrated that they have the capacity to activate the host's innate immune system through the stimulation of neutrophils and macrophages, which then release their chemical mediators (Tada et al. 2002; Noleto et al. 2004; Ishida-Okawara et al. 2007; Torrecillas et al. 2007). They can also trigger a powerful humoral immune response in the host by stimulating production of specific antibodies (Hassan and Ragab 2007; Oliveira et al. 2009).

The aim of this study was to evaluate the production of specific antibodies in Swiss mice treated with cell-wall mannoproteins extracted from the yeast *Saccharomyces uvarum* and inoculated with sheep red blood cells.

MATERIALS AND METHODS

Obtaining the mannoproteins (MP)

Initially, MPs were extracted from the cell walls of *S. uvarum* following autolysis of the yeast cell suspension. Subsequently, the material was centrifuged at 3500 g for 5 min at 10°C, the supernatant was discarded, and the MPs were extracted with distilled water at 95°C for 9 h at 100 rpm, in accordance with the method of Costa (2008). The material was again centrifuged at 3500 g for 5 min at 10°C, and the supernatant was recovered and added to ethanol to precipitate the MPs at 4°C for 12 h. The precipitate was dialyzed in distilled water at 4ºC for 48 h. After dialysis, the MPs were lyophilized and stored at room temperature.

Chemical composition of the MPsThe composition of the MPS was first characterized in accordance with the standards of the Adolfo Lutz Institute (Instituto Adolfo Lutz 1985). The sugar composition was determined by High Performance Liquid Chromatography (HPLC) as described by Moreira et al. with modifications (Moreira et al. 1998). The samples were previously prepared using a pre-column (CarboPac PA1 - Dionex) and an ion exchange column (anion high performance, 10 mm x 250 mm x 4 mm) (CarboPac PA1 - Dionex) at 28ºC in a thermostat-controlled oven (Waters) with a mobile phase consisting of 1.4 mmol NaOH/L, a NaOH regenerative stage of 300 mmol/L and a flow rate of 1.0 mL/min and with a pulse amperometric electrochemical detector.

To estimate the relative molecular mass of the MPs, 12% polyacrylamide gel electrophoresis with SDS (SDS-PAGE) was performed, as proposed by Towbin and Gordon (1984), using a solution of MPs (50 μg/mL).

Experimental design

The immunostimulatory properties of the MPs were investigated in 40 male Swiss mice weighing between 25 and 30 g. The mice were placed in 5 groups (A, B, C, D and E) of 8 animals each. All mice were challenged intraperitoneally (i.p.) with 2% sheep red blood cells (SRBC) prior to treatment and on day 28 of the experiment. In group A, the mice were inoculated i.p. with 200 μL of phosphate buffered saline (PBS, pH 7.2), and the mice in groups B and C received i.p. injections of 200 μL of a solution of 1000 μg or 100 μg of MP in PBS (pH 7.2), respectively, at the same time as the challenge with SRBCs (2%). Mice from groups D and E received i.p. injections of 200 μL of a solution of 100 μg of MP in PBS (pH 7.2) 6 or 24 h before inoculation with SRBCs (2%), respectively. On days -1, 7 and 35, samples of blood were obtained from the mice, and the plasma was separated to determine antibody titers. Mouse thymuses, spleens and livers were collected at the end of the experiment, and the mean weights of the organs were compared across treatments.

Humoral Immune Response

Total antibody (Total Ig) titers were determined by hemagglutination of the SRBCs, as described by Hudson and Hay (1989). Antibody titers of IgG were determined in the presence of β-

mercaptoethanol (0.2 M). IgM titers were calculated by subtracting IgG from the total Ig titers. All results are expressed as Log_2 of the reciprocals of the last dilutions with positive agglutination.

Statistical Analysis

One-way ANOVA followed by the Tukey test was used to compare means on the same day and to compare the mean weights of organs. Student's t test was used to compare the mean of each treatment on different days. Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Analysis of the chemical composition of the MPs revealed that they were 78.0% carbohydrate—mannose (86.12%), glucose (13.42%) and xylose (0.46%) —18.98% protein, 2.58% ash and 0.33% lipids. Moreover, analysis by SDS-PAGE revealed that a protein with a molecular mass of approximately 52 kDa was predominant (data not shown). These results suggest that the MPs obtained in this work are structurally similar to MP65 of *C. albicans*, which is recognized as a potent stimulator of the immune system (Torosantucci et al. 1993).

Several studies show that fungi MPs have an immunomodulatory action in mammals and birds (Torosantucci et al. 1993; Pietrella et al. 2001; Cao et al. 1998; Mencacci et al. 1994; Garner and Domer 1994). Mice treated with an extract of MPs 24 h before or during a challenge with *C. albicans* developed suppression of delayed hypersensitivity reaction with no reduction in the resistance of the mice to *C. albicans*, suggesting that the MPs may be associated with a protective immune response (Garner and Domer 1994). Furthermore, observation verified that an extract rich in 65 kDa MPs from *C. albicans* has the ability to stimulate a lymphoproliferative response with a pattern of Th1 and protection against C. *albicans* (Torosantucci et al. 1993; Mencacci et al. 1994). The characterization of this 65-kDa MP resulted in the identification of peptides related to the lymphoproliferative response that have significant homology with the MPs of *Saccharomyces cerevisiae* (Gomez et al. 2000). Another MP of 58 kDa with immunomodulatory activity has been identified in *C. albicans*. It belongs to a family of fungal immunogenic MPs and stimulates a high production of specific antibodies (Viudes et al. 2001). The main mechanism by which the MP produces the modulation of the immune response is related to mannose receptors present on dendritic cells (Mansour et al. 2006; Fernández et al. 2005). However, the protein portion of mannoproteins has an intrinsic capacity to stimulate a potent immune response (Pietrella et al. 2008; La Valle et al. 2000).

To investigate the immunomodulatory effects of the *S. uvarum* MPs, the primary and secondary immune responses of mice treated with different doses of *S. uvarum* MPs were analyzed. Titers of total Ig, IgG and IgM in mice treated with the MPs of *S. uvarum* are shown in Table 1. There was a significant difference in total Ig titers between treatments after the first challenge with SRBCs. Mice that received 100 μg of MP on the same day or 24 hours before the SRBC inoculation (groups C and E) presented total Ig titers higher than mice receiving the other treatments. In the secondary immune response, total Ig titers in MP-treated mice differed significantly from the total Ig titers of mice not treated with MPs. Moreover, mice treated with 100 μg of MP on the same day or 24 hours before the SRBC challenge (groups C and E) presented higher IgG titers than mice subjected to other treatments. In contrast, no significant differences were observed in IgM or IgG titers between the different treatments on day 7 or in IgM titers on day 35 of the experiment.

The immunostimulatory effects of the MPs on the humoral immune response observed in this study are in agreement with those reported by Paulovičová et al. (2005), who administered cell-wall mannans conjugated with human serum albumin to mice at intervals of two weeks and observed a slight increase in IgM after the first and second applications of this combined solution, with a reduction in IgM titers following the third application and a significant increase in IgG titers. Likewise, female turkeys fed diets containing mannan oligosaccharides showed higher levels of IgM in relation to IgG in their primary immune responses 7-14 days after the first challenge with SRBCs. One week after the second challenge, the birds showed higher production of specific IgGs, a reversal of the previous result (Ferket et al. 2002). Laying hens have shown increased serum antibody titers after first and second challenges with SRBCs when fed diets supplemented with mannans from the cell walls of yeast added to protein molecules

because these are recognized by host T cells (Hassan and Ragab 2007).

Table 1 - Titers of total immunoglobulin (Total Ig), IgM and IgG from mice administered different amounts of mannoproteins intraperitoneally on days 7 and 35 of the experiment. The results are expressed as the means ± standard deviations of the reciprocals of the plasma titers.

Groups	7 days			35 days		
	Total Ig	IgM	IgG	Total Ig	IgM	IgG
A	3.67 ± 1.5	2.33 ± 1.2	1.33 ± 1.2	7.33 ± 0.8	1.17 ± 0.7	6.17 ± 1.4
B	4.33 ± 0.5	3.17 ± 0.7	1.17 ± 1.1	9.17 ± 0.4[#]	1.83 ± 0.4	7.33 ± 0.5
C	5.50 ± 0.8[#]	2.83 ± 0.7	2.67 ± 0.5	9.33 ± 0.8[#]	1.67 ± 0.5	7.67 ± 0.8[#]
D	4.67 ± 0.5	2.50 ± 0.5	2.17 ± 0.9	8.83 ± 0.4[#]	1.50 ± 0.5	7.33 ± 0.5
E	5.50 ± 0.5[#]	3.50 ± 1.3	2.00 ± 1.6	9.67 ± 0.5[#]	1.50 ± 0.5	8.17 ± 0.4[#]

A = positive control; B = 1000 μg mannoprotein; C = 100 μg mannoprotein; D = 100 μg mannoprotein 6 h before challenge with 2% SRBCs; E = 100 μg mannoprotein 24 h before challenge with 2% SRBCs. [#] Means differ from group A by the Tukey test ($P < 0.05$).

The mean weights of the thymuses, spleens and livers of the mice treated with MPs are presented in Table 2. No differences were observed between the mean weights of the organs of mice treated with different concentrations of MP and the control group. However, a trend towards increased weight of the thymus in mice treated with 100 μg of MP 24 h before infection (Group E) was observed, as was as an increase in the weights of the spleens and livers for all mice treated with MPs.

Table 2 - Mean organ weights of mice treated with different levels of mannoproteins intraperitoneally on day 35 of the experiment.

Groups	Thymus (g)	Spleen (g)	Liver (g)
A	0.064 ± 0.02	0.110 ± 0.01	1.923± 0.22
B	0.065 ± 0.01	0.149 ± 0.04	2.187 ± 0.25
C	0.058 ± 0.10	0.171 ± 0.21	2.338 ± 0.21
D	0.060 ± 0.02	0.118 ± 0.05	2.090 ± 0.41
E	0.080 ± 0.02	0.141 ± 0.03	2.128 ± 0.13

A = positive control; B = 1000 μg mannoprotein; C = 100 μg mannoprotein; D = 100 μg mannoprotein 6 h before challenge with 2% SRBCs; E = 100 μg mannoprotein 24 h before challenge with 2% SRBCs.

The findings of this study corroborate those reported by Morales-López et al. (2009), who fed chickens diets containing 95 or 190 mg/kg of MP and observed no significant increases in the weights of the thymuses, spleens or livers of the birds in comparison with other treatments, but these values were higher than those of the control group. However, turkeys fed this polymer and challenged with pathogenic bacteria showed a significant increase in liver weight when compared to control birds, as well as a slight swelling of the spleen, indicating that these organs play an important role in the inflammatory immune response and are involved in lymphocyte activation and antibody production (Ferket et al. 2002).

The results obtained in this work show that MPs from *S. uvarum* are able to stimulate primary and secondary immune responses against a specific antigen without significantly altering the weights of the organs examined. Their ability to stimulate the humoral immune response against unrelated antigens may be important for the development of safer and more efficient adjuvants than those currently in use. They may also have other biomedical properties, such as inhibitory activity against tumors, hematopoietic and radioprotective effects (Križková et al. 2001; Drábiková et al. 2009), antioxidative activity (Križková et al. 2001; Drábiková et al. 2009; Kogan and Kocher 2007), stimulation of nitric oxide production (Noleto et al. 2004) and prevention or elimination of colonization by enteropathogenic bacteria in the gastrointestinal tract of the host (Torrecillas et al. 2007; Kogan and Kocher 2007). Furthermore, the disposal of waste generated in various industrial

activities is an important environmental problem (Tayibi et al. 2009). Several measures have been developed to reduce the amount of industrial waste, including the use of technology to produce less polluting waste, proper treatment of waste before its disposal in the environment and recovery of waste for the manufacture of a new product (Papanikolau et al. 2007; Zverlov et al. 2006). This is especially important in the case of waste generated by fermentation processes where the microorganism used can be processed to obtain biologically active components with high added values (Lamoolphaka et al. 2006). Therefore, studies involving the MPs of *S. uvarum* could result in the generation of a product with a high added value from an industrial waste that is currently sold at a low price or even discarded directly into the environment, causing an undesirable environmental impact.

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