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The glycoprotein (18 kDa) isolated from *Lactobacillus plantarum* L67 suppressed ß-hexosaminidase, histamine, and the expression of TNF-α and IL-4 in the BPA-stimulated RBL-2H3 cells

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ABSTRACT

This study investigated the inhibitory effect of the 18 kDa glycoprotein isolated from *Lactobacillus plantarum* L67 on the bisphenol A-mediated inflammatory reaction in RBL-2H3 cells. The RBL-2H3 cells were treated with bisphenol A (50 μmol) and the glycoprotein isolated from *L. plantarum* L67 (5–100 μg/ml) for 30 min. Subsequently, the release of histamine and β-hexosaminidase into the medium and the intracellular Ca^{2+} levels were measured. The activities of PKC and iNOS were measured by western blotting. In addition, the gene expression levels of IL-4 and TNF-α were measured by qRT-PCR. In conclusion, the results demonstrated that the degranulation of the BPA-stimulated RBL-2H3 cells and their histamine release were inhibited by the glycoprotein of *L. plantarum* L67; concurrently, the levels of intracellular Ca^{2+}, the activity of iNOS, and the expression of TNF-α and IL-4, two allergy-related cytokines, were also suppressed by the glycoprotein. Taken together, these results indicate that the glycoprotein might help to prevent allergic diseases induced by environmental hormones, such as BPA.

1. Introduction

The probiotics lactobacilli and bifidobacteria are the most frequently and safely used bacteria, even for children and immunocompromised individuals [1]. *Lactobacillus plantarum*, in particular, as a heterogeneous and versatile species that is encountered in a variety of environmental niches, including fermented food products, such as dairy, meat, fish, and vegetables, as well as plant matter. This species has a demonstrated ability to survive gastric transit and to colonize the intestinal tract of humans and other mammals. More recently, we discovered that *L. plantarum* L67 isolated from infant feces has a high survival rate in low pH conditions. *L. plantarum* exerts various biological functions, including antitumor, anticoagulant, antiviral, immune regulatory, anti-inflammatory, anti-diabetic, anti-oxidant and free radical scavenging effects [2–5].

Bisphenol A (BPA) is readily find in plastic products, such as toys, drinking containers, dental sealants, water pipes, and food and beverage containers. In addition, it is well-known that endocrine-disrupting chemicals, such as xenoestrogen, can impact the immune system of both humans and animals [6,7]. Many studies have demonstrated that BPA can affect the immune system by inducing the production of cytokines and immunoglobulins. For example, animal data suggested that BPA can modulate the levels of IFN-γ and IL-10 and increase the production of IL-4 and IgE [7–9]. It has been reported that many environmental chemicals are readily encountered in daily life and can alter endocrine and immune system function [10]. Thus, BPA can induce allergic disorders, including atopic dermatitis, allergic rhinitis, and bronchial asthma [11,12].

During the type I immune response, the activation of the immunoglobulin E (IgE)-mediated FceRI receptor, known as the high-affinity IgE receptor, on the plasma membrane of mast cells and basophils leads to the release of ß-hexosaminidase, a common marker of degranulation, as well as various mediators of allergic responses, including histamine, cytokines, prostaglandins and leukotrienes [13]. In particular, IL-4 can be produced by mast cells and induce an allergic response through the subsequent stimulation of additional inflammatory mediators [13,14]. Stimulated mast cells are characterized by the phosphorylation of protein kinases and the production of Reactive Oxygen Species (ROS). These actions are then followed by the activation of mitogen-activated protein kinases (MAPKs) and the redox-sensitive activator protein (AP)-1, thereby producing numerous inflammatory mediators. Therefore, the modulation of degranulation-associated factors in mast cells is a mechanism to regulate immune responses [9].

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Recently we discovered that proteins isolated from *L. plantarum* L67 display several biological activities, including antioxidative effects, anti-tumor effects, and, preliminarily, the detoxification of cadmium [3,15]. Thus, we were interested in whether these proteins could prevent the allergic reactions caused by environmental hormones, such as BPA.

First, the 18 kDa glycoprotein was isolated from *L. plantarum* L67; second, the production of inflammatory mediators by the BPA-stimulated RBL-2H3 cells in the presence of the glycoprotein was evaluated.

The inflammatory response was measured with biochemical analyses of ß-hexosaminidase and histamine release, western blotting for the activity of iNOS, and qRT-PCR for the allergic signals TNF-α and IL-4.

2. Materials and methods

2.1. Chemicals

Bisphenol A, penicillin G, and streptomycin were obtained from Sigma (St. Louis, MO, USA). DMEM and fetal bovine serum (FBS) were purchased from Gibco BRL (Grand Island, NY, USA). Other chemicals and reagents were of the highest analytical grade available.

2.2. Bacterial strain and culture

L. plantarum L67 (Accession Number: KR336551) from infant feces was selected according to Song et al. [16]. For the identification of *L. plantarum* L67, its biochemical properties were first examined with an API 50CHL kit (BioMerieux, France) and later with 16s rDNA sequencing data, as described by Song et al. [16]. *L. plantarum* L67 was grown in MRS broth at 37 °C for 18 h [17]. The bacterial cells were separated by centrifugation at 3000 × *g* for 15 min at room temperature. Subsequently, the pelleted cells were washed twice with sterile saline (0.85% sodium chloride) and resuspended in 1 ml of 10% skim milk; the suspension was stored at −80 °C until further use.

2.3. Preparation of the glycoprotein isolated from plantarum L67

For the protein preparation, 10 ml of bacterial cultures was harvested after 18 h of growth at 37 °C. The pellets were collected by centrifugation (12,000 × *g*, 4 °C) and washed three times with sterile, deionized water. The washed cells were resuspended in 10 g/l of an SDS solution and boiled for 10 min. The supernatants were harvested after centrifugation (12,000 × *g*, 4 °C, 15 min) and dialyzed against distilled water at 4 °C. The supernatants were dried with a freeze-dryer and stored at −70 °C. The protein concentrations were measured using the Bradford assay [18]. We analyzed the protein (100 mg/ml) using SDS-PAGE with a 14% polyacrylamide mini-gel containing 0.1% SDS and a Mini-PROTEAN II electrophoresis cell (Bio-Rad, California, USA) at 110 V and 30 mA. The L67 protein was confirmed by staining with Schiff's reagent [19].

2.4. Cell culture

The RBL-2H3 cells were provided by the Korea Cell Line Bank (KCLB, Seoul, Korea). The cells were grown in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C and in an atmosphere containing 5% CO_2. The medium was replenished twice each week. The cells (2.3×10^6 cells/ml) were seeded into 6-well plates. The cells were treated with 50 μmol BPA and 5–100 μg/ml L67 protein for 30 min [20].

2.5. Cytotoxicity of the L67 total protein in the RBL-2H3 cells

The cellular cytotoxicity induced by the treatment with the L67 protein was measured using a colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) [21]. The cells (1×10^6/ml) were treated with L67 protein (100 μg/ml). Subsequently, 2 μl of an MTT solution (5 mg/ml in PBS stock solution) was added into each well. The cells were further incubated at 37 °C for 4 h. After the complete removal of the medium, 50 μl of acidic isopropanol was added to each well, and the plates were analyzed at 560 nm using a SpectraCountTM ELISA reader (Packard Instrument Co., Downers, IL, USA).

2.6. Pronase E and $NaIO_4$ treatments

The glycoprotein isolated from *L. plantarum* L67 were treated with either Pronase E [22] and NaIO4 [23] to destroy the protein and the carbohydrate portions of the whole glycoprotein isolated from *L. plantarum* L67, respectively. Briefly, the glycoprotein isolated from *L. plantarum* L67 were incubated with 0.4 mg of Pronase E at 30 °C in 4 ml of 0.1 M Tris–HCl buffer (pH 8.0) containing 50 mM CaCl2. After 36 h of incubation, an additional 0.2 mg of Pronase E was added and the incubation was continued for another 36 h. Thereafter, the reaction mixtures were heated at 100 °C for 10 min to inactivate the Pronase E, and then dialyzed against PBS before being passed through a Sephadex G-150 column. In addition, the glycoproteins were incubated with 100 μl of 0.1 M NaIO4 at 25 °C for 4 h, at which point 250 μl of 20% ethylene glycol was added before the samples were dialyzed and eluted as described above. These samples glycoprotein isolated from *L. plantarum* L67 were treated with a deactivation agent (Pronase E and NaIO4) in order to test their inhibit activities of β-hexosaminidase secretion and histamine release.

2.7. β-hexosaminidase secretion assay

The cells were treated with BPA (50 μmol) or cotreated with BPA (50 μmol) and the L67 protein (5–100 μg/ml) for 30 min. At the end of the incubation period, aliquots (20 μl) of the medium were incubated with equal volumes of 1 mmol *p*-nitrophenyl-*n*-acetyl β-D-glucosaminide in 0.1 mol sodium citrate buffer (pH 4.5) at 37 °C for 1 h. The reaction was terminated by adding 200 μl of a stopping buffer (0.1 mol Na2CO3/NaHCO3, pH 10.0). The absorbance of each sample was measured at 405 nm with a microplate reader (Molecular Devices, Sunnyvale, CA).

2.8. Histamine release assay

The cells were treated with BPA (50 μmol) or cotreated with BPA (50 μmol) and the L67 protein (5–100 μg/ml) for 30 min.

The histamine content was measured by a fluorometric assay [24]. To determine the levels of total cellular histamine, the RBL-2H3 cells (1.0×10^6 cells) were suspended and sonicated in Tyrode buffer. Next, the cell lysates were centrifuged at 10,000 × *g* for 10 min, and the supernatants were harvested. The supernatant (2 ml) was first mixed with 0.75 g of NaCl and 0.5 ml of 1 N NaOH and then with 5 ml of a 3:2 (v/v) mixture of *n*-butanol and chloroform for 5 min. The solution was subsequently centrifuged for 5 min at 300 × *g*, and 4 ml of the upper organic solvent layer was recovered. The recovered layer was mixed with 2 ml of *n*-heptane and 1.5 ml of 0.1 N HCl. After centrifugation at 300 × *g* for 5 min, 1 ml of the lower HCl layer was recovered and mixed with 0.15 ml of 1 N NaOH, and the subsequent solution was mixed with 0.1 ml of 0.2% *O*-phthalaldehyde dissolved in methanol for 5 min at room temperature to yield a fluorescent product. The reaction was terminated by adding 0.14 ml of 0.5 N H_2SO_4, and the fluorescence