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Characterization of Maillard-type lysozyme-galactomannan conjugate having immune-enhancing effects

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ABSTRACT

In the present study, lysozyme-galactomannan conjugate (LGC) was fractionated by ion-exchange chromatography, the immune activity of the fractions was confirmed, and a structural analysis of the glycoprotein was performed. A high-molecular-weight fraction of LGC (H-LGC), was characterized by using a method using matrix-assisted laser desorption/ionization time of flight mass spectrometry. The glycated site of H-LGC was determined to be the lysine (Lys)115 residue. In addition, about 1 mol of galactomannan (G) was linked to 1 mol of lysozyme (L) in LGC based on the binding weight ratio. Conjugation of L and G reduced the aggregation of particles, resulting in a monodispersion based on measurement of dynamic light scattering. LGC in solution showed heterogeneous shapes with a mean size of 337 nm. Therefore, we suggest that LGC improves the immune-enhancing activity as G conjugates the site of Lys115 on L, and provides higher solubility with reduced aggregation for the industrial use of LGC as a food constituent.

1. Introduction

Maillard-type protein-carbohydrate conjugates can be produced by allowing the carbonyl group of the carbohydrate to react with the ε-amino group of the lysine (Lys) residue or *N*-terminal amino group of the protein (Nakamura, Kato, & Kobayashi, 1992). Dry heating is often adopted to prepare Maillard-type protein-polysaccharide conjugates under controlled temperature and relative humidity (RH) conditions, and many researchers have reported that protein-polysaccharide conjugates produced under these controlled conditions show new functional and biological properties (Akhtar & Dickinson, 2007; Diftis & Kiosseoglou, 2003; Xu & Yao, 2009). Kato et al. reported that protein-polysaccharide conjugates prepared by spontaneous Maillard reaction (MR) under controlled dry heating revealed significant improvements of the functional attributes of proteins, such as solubility, heat stability, and emulsifying properties (Ames, 1992; Kato, Minaki, & Kobayashi, 1993; Kato, Sasaki, Furuta, & Kobayashi, 1990; Nakamura et al., 1992). In addition, lysozyme-galactomannan conjugate (LGC) produced by dry heating at 60 °C for 2 weeks at 79% RH has been reported to show protection against *Edwardsiella tarda* infection in carp (*Cyprinus carpio* L.) (Nakamura, Gohya, Losso, Nakai, & Kato, 1996). Lysozyme-dextran conjugates prepared by dry heating at 60 °C for 3 weeks at 78.9% RH had antibacterial effects against *Escherichia coli*, and showed excellent emulsifying properties (Nakamura, Kato, & Kobayashi, 1991).

Galactomannan (G) is a polymer of d-mannose with d-galactose found in legumes, bacteria and yeast (IFIS, 2009). Especially the G of locust bean gum contains about 20% galactose, and mannose to galactose ratio is around 3.5:1. G has effects of immune-modulatory (Badia et al., 2012) and reducing inflammation like inflammatory bowel diseases (Galvez, Rodríguez-Cabezas, & Zarzuelo, 2005). G is suitable for food applications such as bakery products, noodles, beverages, ice cream and yogurt as food additive (Barak & Mudgil, 2014). Lysozyme (L), widely distributed in food (i.e., milk, egg and fish) is a key self-defense molecule in innate immune system (Benkerroum, 2008; Saurabh & Sahoo, 2008). L has effects of antibacterial toward gram-positive bacteria by attacking peptideglycan and enhancing phagocytosis (Benkerroum, 2008). In a previous study, we successfully built a protein-polysaccharide Maillard model system to improve immune properties of conjugates, enabling evaluations of the

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immune-enhancing effects of a Maillard-type LGC produced in the dry-heating model system. LGC significantly induced nitric oxide, and the expression of tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-8 on murine macrophages (Raw 264.7 cells), which are primary immune defense cells. The induction of these immune-modulating factors was found to occur via the JNK, ERK and NF-κB pathways (Ha et al., 2013). It is important that LGC with immune-modulating effects is structurally characterized, because the structure of LGC may help elucidate the relationship between LGC and macrophage by modification of the recognition site of protein leading to enhanced immune activity of its conjugated protein. In the present study, therefore, we characterized LGC, focusing on a structural analysis of the new glycoprotein.

As a follow-up study of the previous finding (Ha et al., 2013), we attempted (a) to fractionate LGC from Maillard reaction products (MRPs) formed in our Maillard model system under controlled conditions (dry heating, 60 °C for 7 d, 79% RH) by ion-exchange chromatography, (b) to investigate the immune activity of the fractions, (c) to characterize the conjugated positions of LGC by comparing peptides from L and a high-molecular-weight fraction of LGC (H-LGC) using MALDI-TOF MS after trypsin digestion, and (d) to confirm NO production of the glycated peptide. In addition, the size distribution of LGC was evaluated indirectly by measuring dynamic light scattering.

2. Materials and methods

2.1. Materials

High-glucose Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin streptomycin was purchased from Gibco (NY, USA). G from locust bean gum of *Ceratonia siliqua* seeds (a molecular weight of approx. 310 kDa), L from chicken egg white, lipopolysaccharide (LPS), OPA (Sigma-Aldrich, St. Louis, USA), phenol and N^{ε}-Acetyl-l-Lys were purchased from Sigma-Aldrich (MO, USA). A BCA protein assay kit was purchased from Thermo Scientific (IL, USA). HiTrap CM-Sepharose Fast Flow columns were purchased from GE Healthcare (Uppsala, Sweden).

2.2. Glycation of lysozyme with galactomannan

L, lysozyme and G, galactomannan were mixed in distilled water (DW) at a weight ratio of 1:5, and then lyophilized. The dried sample was incubated for 7 d at 60 °C and 79% RH using saturated KBr solution in a desiccator according to a previously published method (Enomoto et al., 2009). For the determination of LGC formation, the LGC was dissolved in DW, and used supernatant of LGC adjusted a protein concentration of 500 μg/mL. Because LGC is difficult to dissolve, LGC dissolved in DW was sufficiently for 30 min, centrifuged at 10,000*g* for 20 min to remove undissolved materials, and the supernatant was filtered through a 0.45-μm filter (Durapore PVDF, Millipore, Bedford, MA). The protein content of the supernatant was determined by BCA assay (Smith et al., 1985).

2.3. Determination of LGC formation

2.3.1. Determination of the degree of glycation

MRPs were reported to generate the fluorescent compounds, and it could be measured at the wave lengths of excitation and emission (Dyer et al., 1993). The fluorescence of LGC was measured at 370 nm excitation and 440 nm emission wavelengths using a VICTOR3 spectrofluorometer (Perkin Elmer, USA) (Nagaraj & Monnier, 1995). Also, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the method of Laemmli (Laemmli, 1970) using 15% Tris-glycine-SDS-PAGE gel (Komabiotech, Seoul, South Korea) for determining high molecular weight formation of protein. Each sample and the control were mixed with 5× SDS buffer (25% [v/v] glycerol, 2% [w/v] SDS, 5% [v/v] β-mercaptoethanol, 0.025% [w/v] bromophenol blue and 60 mM Tris-HCl, pH 6.8) and boiled for 5 min. The protein (1.2 μg) was loaded into each well, and then electrophoresed at 100 V for 3 h. The finished gel was stained with Coomassie Brilliant Blue R250 (Kojima Chemicals Co. Ltd, Kyoto, Japan). Smart Color Protein Marker (Elpisbio, Deajeon, South Korea) was used as the molecular weight standard.

2.3.2. Determination of the free amino groups in LGC

The free amino groups of L and LGC were determined using a modified fluorogenic OPA assay (van de Lagemaat, Manuel Silván, Javier Moreno, Olano, & Dolores del Castillo, 2007). The OPA reagent was prepared by dissolving 40 mg of OPA in 1 mL of methanol, 50 mL of 0.1 M sodium borate buffer (pH 10), 200 μL of mercaptoethanol and 1.5 mL of 20% (w/v) SDS solution. The mixture was added to an equal volume of 12% (w/v) SDS solution, and stirred overnight at room temperature. Then, 300 μL of OPA reagent was added to 100 μL of each sample solution and incubated for 5 min at room temperature. Fluorescence was measured at 360 nm excitation and 460 nm emission wavelengths using a VICTOR3™ spectrofluorometer (Perkin Elmer, USA). A calibration curve was obtained using 10–250 μM N^{ε}-acetyl-l-Lys (Sigma-Aldrich, St. Louis, USA) as a standard.

2.4. Immune-enhancing effect of LGC

2.4.1. Cell culture and sample preparation

A murine leukemia cell line, Raw 264.7, was purchased from the Korean cell bank (Seoul, Korea). Cells were grown in high-glucose DMEM with 10% FBS, 100 U/mL of streptomycin, and 100 U/mL of penicillin (Gibco, BCL, Burlington, Ontario, CA) at 37 °C in a 5% CO_2 atmosphere. The sample for cell treatment was prepared by dissolving LGC in medium with mixing for 30 min, centrifuging at 10,000 x *g* for 20 min to remove undissolved materials, and then filtering the supernatant with 0.2 μm filters.

2.4.2. NO measurement

Briefly, RAW 264.7 cells were seeded in 96-well plate at the density of 5×10^5 cells/mL. After seed for 24 h, the cells were treated with LGC, and incubated at 37 °C in a 5% CO_2 atmosphere for 24 h. To measure the concentration of NO produced during a 24-h incubation period, the stable conversion product of NO, nitrite (NO_2^-), was measured using the Griess method (Schulz, Kerber, & Kelm, 1999). Supernatant (50 μL) from each well was transferred to another 96-well plate, and 100 μL of the Griess reagent (1:1 mixture [v/v] of 1% sulfanilamide and 0.1% [w/v] naphthylethylenediamine dihydrochloride in 5% [v/v] H_3PO_4) was added to each well. The solution was incubated at 25 °C for 10 min. The absorbance at 540 nm was measured with a microplate reader (EL-808, BioTek, Seoul, Korea). Control (CON) was the group without treatment, and LPS was the positive control with treatments of 0.5–10 ng/mL of LPS.

2.4.3. Total RNA isolation, cDNA synthesis and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Briefly, RAW 264.7 cells were seeded at the density of 4.5×10^5 cells/mL in 12-well plate. After seeding for 24 h, the cells were treated with LGC, and incubated at 37 °C in a 5% CO_2 atmosphere for 24 h. Total RNA was isolated using Trizol reagent (invitrogen, NY, USA) according to the manufacturer's instructions for cells in a monolayer. The first-strand cDNA, primed by an oligo-primer, was prepared from the total RNA (3 μg) using a 1st Strand