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Significant reduction in allergenicity of ovalbumin from chicken egg white following treatment with ascidian viscera *N*-acetylglucosaminidase

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ABSTRACT

Ovalbumin (OA) is the most abundant ingredient of chicken egg-white allergenic proteins. In the present study we investigated the possibility of reducing OA allergenicity by treatment with a natural protein exhibiting *N*-acetylglucosaminidase (NA) activity. Ascidian is cultivated as a food resource in northeast Asia. The ascidian viscera NA (AVNA) with almost no other exoglycosidases or proteolytic enzymes was isolated by applying size-exclusion chromatography to a protein precipitate of ascidian viscera. Intact OA was mixed with AVNA containing 0.2, 1.0, and 5.0 Units of NA. Anion-exchange chromatography was then used to isolate OA from AVNA-treated OA. The electrophoretic patterns and *N*-glycans of each isolated OA from AVNA-treated OA (iOA) were analyzed, and the terminal *N*-acetylglucosamines of iOA were selectively cleaved with no other degradation occurring. A competitive indirect enzyme-linked immunosorbent assay using rabbit anti-OA sera was performed to investigate the allergenicity of iOA, which was found to be significantly reduced depending on the increased NA activity compared to that of intact OA. These results indicate that OA allergenicity was reduced using a simple and mild treatment process with AVNA, and suggest that ascidian NA is an efficient natural protein for reducing the allergenicity of OA without requiring the use of harsh physical treatments or chemical conjugation.

1. Introduction

Food allergy involves an adverse immune response to one or more specific foods. Egg is one of the major allergens, with approximately 1.5% of children and 0.2% of adults being allergic to egg proteins in North America [1]. Egg allergy is caused by major egg allergenic proteins, such as ovalbumin (OA), ovotransferrin, ovomucoid, and lysozyme [2], of which OA is the most abundant ingredient, constituting about 54% of the total egg-white proteins [3]. OA allergy is associated mainly with an IgE-mediated type I hypersensitivity that is characterized by an increased production of IgE antibodies and Th2 cytokines [4].

Some results related to reducing OA allergy have been reported recently. Physical methods such as heat treatment or gamma irradiation of OA decreases its allergenicity. OA heated for 10 min at 70 °C showed irreversible changes in secondary structure, and immunogenic properties were formed leading to the shift from Th2- to Th1-type responses as compared to native OA [5]. Treating OA at 100 °C for 5 min resulted in a total loss of allergenicity [6], while exposing OA to gamma radiation at 50 and 100 kGy decreased Th2 cytokine levels in OA-sensitized mice [7]. In addition, the chemical conjugation of OA with mannose (Man) or glucomannan [8,9] or OA–interleukin-12 fusion protein [10] also reduced its allergenicity. However, the glycated form of OA is more immunogenic and allergenic than intact OA [11].

Our recent study showed that cleaving terminal *N*-acetylglucosamine (GlcNAc) residues of OA glycan reduced IgE production

Abbreviations: AVE, ascidian viscera extract; AVNA, ascidian viscera *N*-acetylglucosaminidase; AVPP, ascidian viscera protein precipitate; ciELISA, competitive indirect enzyme-linked immunosorbent assay; GlcNAc, *N*-acetylglucosamine; HPLC, high-performance liquid chromatography; IC_{50}, half-maximal inhibitory concentration; iOA, isolated ovalbumin from AVNA-treated ovalbumin; Man, mannose; NA, *N*-acetylglucosaminidase; OA, ovalbumin; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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and Th2 cytokine secretion in a mouse model [12]. Namely, the cleavage of the terminal GlcNAc from intact OA dramatically affected the OA allergenicity, and this result suggests that the removal of the terminal GlcNAc is an efficient way of reducing the allergenic risk of OA. Although the underlying mechanism is not clear, the glycosylation pattern in a glycoprotein is one of the important factors influencing the effects of glycoprotein allergens [13], and the glycosylation in glycoprotein may affect several properties of its protein such as stability, solubility, hydrophobicity, and electrical charge, and also the availability of proteolytic sites of proteins [14–16].

Exoglycosidases are glycoside hydrolase enzymes cleaving the glycosidic linkage of terminal residues of monosaccharide in glycans. These exoglycosidases are used to remove a terminal monosaccharide residue, to determine the sequence of a glycan, or for modifying glycans on glycoproteins [17]. Exo-β-*N*-acetylglucosaminidase (*N*-acetylglucosaminidase; NA) is one of the exoglycosidases that hydrolyze terminal and nonreducing GlcNAc residues [18]. NA is expressed by various organisms and several NAs are well characterized, with their molecular mass and their optimum pH and temperature for enzyme activity having been reported, including those from ascidian (*Halocynthia roretzi*) [19], jack bean (*Canavalia ensiformis*) [20], wheat bran (*Triticum aestivum*) [21], cabbage (*Brassica oleracea*) [22], pea seeds (*Pisum sativum* L.) [23], and shiitake mushroom (*Lentinula edodes*) [24]. All of these NAs have differing protein characterizations and structures. Ascidian is widely available due to it being cultivated as a food resource in the sea to the south of Korea and in northern Japan [25].

The present study investigated the possibility of reducing the allergenicity of OA by the effective application of natural source of NA using ascidian extracts. After comparing the NA activity and other exoglycosidases (galactosidase and mannosidase) in the three parts of ascidian (i.e., viscera, tunic, and remaining parts), NA with a high specific activity was isolated by applying size-exclusion chromatography from ascidian viscera proteins. OA was mixed with this NA for 18 h at 37 °C, and then the OA was isolated using anion-exchange chromatography. The efficiency of cleavage of terminal GlcNAc residues of isolated OA were investigated and compared with that of intact OA. The allergenicity using rabbit anti-OA sera was also investigated and compared with that of intact OA.

2. Materials and methods

2.1. Preparation of ascidian extracts

Ascidian was purchased from Noryangjin Fisheries Market, Seoul, South Korea. Whole ascidian was divided into its viscera, tunic, and remaining parts, and these were separately ground using a grinder, and the homogenates were extracted by stirring with a volume of five parts distilled water to one part homogenate for 18 h at 4 °C. These extracts were centrifuged for 30 min at 3000 × g, and the supernatants were filtered with a 0.45-μm syringe filter, and then stored at 4 °C. The protein concentration was determined with a bicinchoninic acid protein assay kit (Intron Biotechnology, Seoul, South Korea) according to the manufacturer's recommendation with bovine serum albumin as a standard.

2.2. Effects of pH on the NA activity of ascidian extracts

A NA assay kit (Sigma, St. Louis, MO, USA) was used to investigate the activities of exoglycosidases (NA, galactosidase, and mannosidase) according to the manufacturer's instructions with slight modifications. Briefly, substrates such as 4-nitrophenyl *N*-acetyl-β-D-glucosaminide (Sigma), 4-nitrophenyl β-D-galactopyranoside (Sigma), and 4-nitrophenyl α-D-mannopyranoside (Sigma) were dissolved in the following buffers at a concentration of 1 mg/ml: 0.1 M citrate-phosphate buffer (pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, and 7.0), 0.1 M sodium phosphate buffer (pH 7.5 and 8.0), and 0.1 M sodium carbonate buffer (pH 9.0). The 4-nitrophenylate ions released from the substrates were assessed by measuring the absorbance at 405 nm.

2.3. Preparation of ascidian viscera NA from ascidian viscera extract

Ascidian viscera extract (AVE) was precipitated using solid ammonium sulfate added to 80% saturation, and centrifuged at 20,000 × g for 30 min. The precipitated proteins were redissolved and dialyzed in 50 mM sodium citrate (pH 5.0). The extracted proteins (ascidian viscera protein precipitate; AVPP) were subjected to size-exclusion chromatography (Sephadex G-100, 2.5 cm × 30 cm, GE Healthcare, Uppsala, Sweden), and separated with 50 mM sodium citrate (pH 5.0). The ascidian viscera NA (AVNA) was collected, dialyzed, and then stored at 4 °C.

2.4. Preparation of AVNA-treated OA

OA was simply mixed with buffer only (negative control), 0.2 Units of NA (jack bean, Sigma) (OA-NA) (positive control), and three AVNAs treated with 0.2, 1.0, and 5.0 Units of NA (OA-0.2-AVNA, OA-1.0-AVNA, and OA-5.0-AVNA, respectively). These five samples were incubated for 18 h at 37 °C, and then stored at 4 °C.

2.5. Isolation of OA from AVNA-treated OA

OA was isolated from AVNA-treated OA using an anion-exchange column (Resource Q, GE Healthcare). The column was equilibrated with 50 mM Tris-HCl (pH 9.0) and eluted by the same buffer containing 1 M NaCl with a gradient from 0% to 30%. OA fractions were collected and dialyzed against distilled water, and then stored at 4 °C.

2.6. Electrophoretic analysis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli [26] using a 12% or 15% acrylamide slab gel. The gel was stained with Coomassie blue R-250. Molecular mass markers (10–250 kDa) were purchased from Bio-Rad (Hercules, CA, USA).

2.7. N-glycan analysis of isolated OA

The *N*-glycans of the isolated OA from AVNA-treated OA (iOA) were analyzed as described previously [27]. Briefly, iOA was incubated with trypsin and chymotrypsin (Sigma) in 10 mM Tris-HCl (pH 8.0) for 18 h at 37 °C, and *N*-glycans were released by digestion with glycoamidase A (Seikagaku Kogyo, Tokyo, Japan). The released glycans were purified using a graphitized carbon cartridge (Alltech, Deerfield, IL, USA) [28] and fluorescently labeled with 2-aminobenzamide [29]. High-performance liquid chromatography (HPLC) was performed using a TSK-gel amide 80 column (4.6 mm × 250 mm; Tosoh, Tokyo, Japan) on an HPLC device (Alliance 2690, Waters, Milford, MA, USA). Fluorescence was observed using emission and excitation wavelengths of 420 and 330 nm, respectively. The detailed HPLC condition and *N*-glycan analysis method are described in our previous report [30].

2.8. Allergenicity of iOA

The allergenicity of iOA was evaluated by performing a competitive indirect enzyme-linked immunosorbent assay

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