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Caffeic acid-assisted cross-linking catalyzed by polyphenol oxidase decreases the allergenicity of ovalbumin in a Balb/c mouse model

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ABSTRACT

Ovalbumin (OVA) is the most abundant egg white protein, but is also a major egg allergen. Desensitization of OVA may be a good way to control an egg allergy. In this study, caffeic acid-assisted cross-linked OVA catalyzed by polyphenol oxidase (PPO) was prepared, the effect of cross-linking on the allergenicity of OVA was tested in a Balb/c mouse model. Mice were orally sensitized with OVA or cross-linked OVA using cholera toxin as adjuvant. Clinical signs of allergy, specific antibody levels, serum histamine levels, mast cell protease-1 (mMCP-1) concentrations, morphological structure of duodenum, and cytokines were determined after mice were challenged with OVA or cross-linked OVA. Both OVA and cross-linked OVA induced allergic diarrhea in Balb/c mice, however, histological symptoms of small intestine were much milder in mice fed with cross-linked OVA than in those fed with OVA. A tendency toward decreased allergen-specific IgE, IgG, IgG1 and IgG2a levels, as well as serum histamine and mMCP-1 concentration were observed in cross-linked OVA group, accompanied by an inhibition of IL-4, IL-5, IL-13 and IFN-γ production in the stimulated spleen cell. It could be concluded that caffeic acid-assisted PPO-catalyzed cross-linking significantly reduced the potential allergenicity of OVA, but may not completely eliminate it.

1. Introduction

Food allergy is recognized as a common worldwide problem and affects an estimated 6–8% of children and 2–5% of adults (Ballmer-Weber and Fernandez-Rivas, 2008; Nowak-Wegrzyn, 2007). Egg is one of the top 8 most allergenic foods in children and infants, with an estimated incidence of 1.6–3.2% (Eggesbo et al., 2001; Houben et al., 2016; Savage et al., 2016). Nowadays, strict dietary avoidance of eggs and egg-containing foods is the best approach to prevent egg allergy, however, due to the unique functional properties of egg proteins, such as foaming, emulsifying, and gelling, the omnipresence of egg proteins in many food products makes it difficult to avoid (McGowan et al., 2015; Mine, 2002). Accordingly, developing hypoallergenic egg products is still a challenging work.

Enzymatic cross-linking is an innovative non-thermal processing method exploited in food industry for improving food texture and functionality (Verhoeckx et al., 2015). The principle of enzymatic cross-linking is to utilize the enzyme to catalyze the polymerization of proteins primarily through formation of intramolecular and intermolecular cross-links (Djoullah et al., 2016; Giosafatto et al., 2012; Heck et al., 2013; Hiller and Lorenzen, 2009). Transglutaminase, peroxidase, tyrosinases, and laccases are usually the most commonly used for proteins cross-linking (Heck et al., 2013). Recently, cross-linking was demonstrated to change the allergenicity of some allergens. For example, transglutaminase cross-linked sodium caseinate showed a tendency toward decreasingcasein-specific IgE levels (van Esch et al., 2013). Cross-linked Ara h2 decreased its allergenicity after being catalyzed by transglutaminase and polyphenol oxidase, respectively (Wu et al., 2016b, 2017). The cross-linked shellfish allergens by tyrosinase or horseradish peroxidase could alter the morphology of proteins and had a high potential for mitigating allergenicity of the shellfish proteins (Fei et al., 2016; Liu et al., 2017). These studies implied that enzymatic cross-linking may be a new technique to reduce the allergenicity of food allergens.

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Egg contains 10–12% of proteins, and four proteins in egg white-includingovomucoid (OVM), ovalbumin (OVA), ovotransferrin (OVT), and lysozyme (Lys), and two proteins in egg yolk including a-livetin and yolk glycoprotein 42 (YGP42), were recognized as the allergens in egg (Amo et al., 2010; Jacobsen et al., 2008). Among these allergens, OVM, OVA, and Lys were cross-linked by transglutaminase and peroxidase, respectively, and it was indicated that the molecular weight, structure, rheological properties, functional characteristics, digestibility, and potential allergenicity of these proteins had been changed after cross-linking (Ma et al., 2015; Porta et al., 2013; Schuh et al., 2010; Yaghoubi et al., 2007). In addition, laccase was also used to catalyze egg white to prepare cross-linked aggregates (Jiang et al., 2014). However, tyrosinases have yet to be utilized to catalyze the cross-linking of egg white proteins.

Polyphenol oxidase (PPO), also often called tyrosinase, is a copper-containing enzyme which could insert oxygen in a position ortho-to a tyrosine residue or an existing hydroxyl group in an aromatic ring, followed by the oxidation of o-diphenols to their cognate o-quinones (Mayer, 2006; VanGelder et al., 1997). With regard to the cross-linking reaction catalyzed by PPO, the weakly defined three-dimensional structure and unfolded proteins were easier to be cross-linked, while cross-linking of globular proteins was limited (Hellman et al., 2011). However, cross-linking between globular proteins couldbe induced by the addition of low molecular weight phenolic compounds, such as caffeic acid (Jus et al., 2012; Thalmann and Lotzbeyer, 2002). This small-molecule phenolic source acted as an assistant mediator for the cross-linking process, which was oxidized by PPO into an o-quinone derivative, followed by generating caffeic-containing protein-protein cross-links (Chung et al., 2005; Thalmann and Lotzbeyer, 2002).

Previous studies demonstrated that PPO catalyzed cross-linking could decrease the allergenicity of peanut allergens (Chung et al., 2005; Wu et al., 2016b), especially with the assist of caffeic acid (Chung et al., 2005), implying PPO catalyzed cross-linking was an effective way to desensitize food allergens. Accordingly, we hypothesized that the allergenicity of egg white allergens could also be decreased by PPO catalyzed cross-linking, and OVA was chosen to verify this hypothesis because it is the most abundant protein in egg white(Huntington and Stein, 2001) and it is usually used as a model protein for studying antigen-specific immune responses in mice (Basto et al., 2015; Claude et al., 2016; Park et al., 2017). Native OVA has a globular structure (Huntington and Stein, 2001), therefore, in the current study, caffeic acid-assisted cross-linked OVA catalyzed by polyphenol oxidase was prepared. The stability and IgE-binding ability of cross-linked OVA were tested by simulated gastrointestinal digestion and ELISA *in vitro*, and the allergenicity of cross-linked OVA was assessed by allergic clinical signs, pathological examination of intestine, specific antibodies levels, serum histamine levels, mast cell protease-1 (mMCP-1) concentrations, as well as cytokines production in a Balb/c mouse model. This work aimed to evaluate the effect of cross-linking catalyzed by PPO on the allergenicity of egg allergen OVA.

2. Materials and methods

2.1. Materials

OVA with a purity of > 98% was prepared and identified by anion exchange chromatography, SDS-PAGE, and MS, respectively, as previously described (Ma et al., 2009). PPO powders were lab-made from mushroom (*Agaricus bisporus*) according to the method of Wu et al. (2013). Pre-stained protein maker was purchased from MBI Fermentas (Burlington, Canada). All other chemicals were purchased from the Sigma Aldrich Corporation (St Louis, MO, USA).

2.2. Human sera collection

Human sera were collected with informed consent from 8 egg allergy patients from the First Affiliated Hospital of Guangxi Medical University (Guangxi, China). The availability of the sera samples was approved by the internal ethical committee of the hospital. Subjects with a strong history of severe systemic reactions to egg and total egg IgE of at least 100 IU/mL were recruited in this study. All patients were confirmed to have an egg allergy based on physical examination and objective clinical manifestations. A sera pool was prepared according to the method of Wu et al. (2016b). containing sera from the eight patients in equal volumes and used for further analysis.

2.3. Preparation of cross-linked OVA catalyzed by PPO

PPO was dissolved in phosphate buffer (pH 7.0), and the enzymatic activity was measured according to the method of Gawlik-Dziki (Gawlik-Dziki et al., 2008) with some modifications. Caffeic acid was dissolved in ethyl alcohol to be a 100 mmol/L solution. 1 mg of the lyophilized OVA was dissolved in a 50 mmol/L solution of phosphate buffer (pH 7.0), followed by caffeic acid was added and mixed well to the final concentration of 1.0 mmol/L. After that, PPO was added into the mixture, and the final enzymatic activity was 1000 U/mL. Finally, the volume of the reaction system brought to 1 mL with 50 mmol/L solution of phosphate buffer (pH 7.0). The reaction was carried out at 50 °C for 8 h and stopped by heating at 80 °C for 5 min. The cross-linked OVA was frozen and stored at −20 °C until use.

2.4. Dynamic light scattering (DLS) measurement

Determination of the mean particle size of 1 mg/mL OVA and cross-linked OVA was carried out with a BI-200SM dynamic/Static light scattering spectrometer (Brookhaven Instruments Corp., USA) at a wavelength of 532 nm and a scattering angle of 90° at 25 °C according to the method of Shan (Shan et al., 2014).

2.5. In vitro simulated gastrointestinal digestion

Simulated gastrointestinal digestion of OVA and cross-linked OVA was performed as previously described (Amigo-Benavent et al., 2011) with some modifications. Briefly, for the simulated gastric digestion, OVA and cross-linked OVA (1.0 mg/mL) were suspended respectively in simulated gastric solution (35 mmol/L NaCl, pH 2.0, adjusted with 2 mol/L HCl) with porcine pepsin (EC 3.4.23.1, 3440 U/mg, Sigma), at an enzyme: substrate ratio of 1:20 w/w (172 U/mg of protein), and incubated at 37 °C in a shaker for 0, 10, 20, 30, 60 min. The reaction was stopped by raising the pH to 7.0 using 2 mol/L $NaHCO_3$. For the intestinal digestion, the digested protein solution after the 60-min pepsin digestion was shifted to pH 7.0 with the addition of 1 mol/L $CaCl_2$, 0.25 mol/L Bis-Tris (pH 6.5) and a 0.125 mol/L bile salts mixture containing the same molar quantities of sodium taurocholate and glycodeoxycholic acid. After preheating at 37 °C for 15 min, trypsin was added at an enzyme: substrate ratio of 1:3 (w/w) to the mixture. The pH was adjusted to pH 7.5 with 1 mol/L NaOH. After that, the reactions were incubated at 37 °C in a shaker for 5, 10, 30, 60 min, and then stopped by boiling for 10 min at 100 °C in water bath. All protein samples were collected and stored at −20 °C, until analyzed by SDS-PAGE.

2.6. IgE binding assessment

To assess the potential allergenicity of OVA and cross-linked OVA, the IgE binding capacity was measured by indirect Enzyme-Linked Immunosorbent Assay (ELISA) (White et al., 2013; Wu et al., 2016a). The individual wells of a 96-well microtiter plate were coated with OVA and cross-linked OVA samples (100 μL per well, from 0.5 μg/mL to 16 μg/mL) in 50 mmol/L sodium carbonate buffer (CBS, pH 9.6) overnight at 4 °C. The wells were washed thrice with phosphate-buffered saline (PBS, pH 7.4) containing 0.05% Tween 20 (PBST), followed