

RESEARCH ARTICLE

Aspergillus oryzae reduces IgE binding ability of allergenic egg white proteins

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Abstract Egg white proteins are one of the major allergens. The objective of this study was to investigate the effect of *Aspergillus oryzae* cultivation on IgE binding ability of egg white proteins. Effect of *A. oryzae* on egg white proteins was determined using ninhydrin method, SDS-PAGE, ELISA, fluorescence FITC labeling, MALDI-TOF-MS and LC-MS/MS analysis. Adding mycelium of *A. oryzae* ATCC 1011 and 16868 substantially reduced the IgE binding ability of acidified egg white after 24 h incubation. The binding capacity of egg white proteins to IgE in plasma from four egg allergy patients was almost completely lost after incubation with mycelium of ATCC 16868. Results from SDS-PAGE, free amino acid analysis, MALDI-TOF-MS and LC-MS/MS indicated that there was no substantial protein degradation during incubation. Therefore, the reduction of IgE binding ability of egg white proteins during *A. oryzae* treatment was probably due to a loss of ~1700 Da mass including a fragment of the ovomucoid N terminus.

Keywords *Aspergillus oryzae*, egg allergy, egg white proteins, IgE-binding ability, ovomucoid

1 Introduction

Egg whites contain four major egg allergens, ovomucoid (Gal d 1, 11%), ovalbumin (Gal d 2, 54%), ovomucoid (Gal d 3, 12%) and lysozyme (Gal d 4, 3.4%)^[1]. Ovomucoid is now recognized as the dominant egg allergen. Unlike other egg white proteins, ovomucoid is resistant to heat and digestive enzymes. Heating at 80°C diminished the IgE binding ability of ovalbumin^[2], whereas ovomucoid retained its IgE binding ability even after heating at 100°C^[3]. Although most (~60%) egg allergic patients are sensitive to heated egg white, the

majority of those show no reaction to heated ovomucoid-depleted egg white^[4]. Ovomucoid contains three tandem domains. Each domain is homologous to pancreatic secretory trypsin inhibitor and possesses three intra-domain disulfide bonds. This makes the protein resistant to heat denaturation and digestive enzymes^[5,6]. Ovomucoid also comprises about 25% to 30% carbohydrate moieties that contribute to stabilization of ovomucoid^[5,7]. (Using 34 sera from adults who had egg allergies, Aabin et al.^[8] reported that ovomucoid and ovomucoid had higher IgE binding ability than ovalbumin and lysozyme. The greater IgE binding ability of ovomucoid and ovomucoid was also confirmed by Jacobsen et al.^[9] It was estimated that about 35% of egg allergy patients are sensitive to lysozyme^[10].

Antigens have two types of epitopes, conformational and linear. Conformational epitopes are made up of several separated peptide chains, which can be quite distant in the primary sequences but are linked loosely by three-dimensional structures^[11]. Since the secondary or tertiary structures of conformational epitopes are critical for their binding ability to bind to IgE^[12], their destructions due to protein denaturation during thermal processing, irradiation, and/or high pressure treatment may reduce the IgE binding ability^[13]. However, it is difficult to destroy the conformational epitopes in ovomucoid, because their compact structure makes them resistant to heat denaturation. The binding ability of linear epitopes, based on the integrity of their primary sequences, is less affected by heat treatment and partial proteolysis^[11]. Linear epitopes are prevalent in food allergens since they can be presented to immune system after the heating and/or digestion^[12]. The dominant egg allergen, ovomucoid, contains at least eight linear epitopes identified for IgE/T cell^[14].

Many types of food processing, such as heating, high-pressure processing, irradiation, enzymes hydrolysis and fermentation, reduce the IgE binding ability of proteins^[13]. Fermentation is widely used in the food industry. *Aspergillus oryzae*, in the format of koji or *Qu*, has been used for thousands of years for preparing many traditional

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Asian foods, such as soy sauce, sake (a rice wine) and miso (fermented soy seasoning). *A. oryzae* produces polysaccharide hydrolases including glucoamylase, α -amylase, and α -glucosidase, and proteases^[15]. It has been hypothesized that the generation of proteases from *A. oryzae* hydrolyzes food proteins and alters their IgE binding ability. It was reported that miso is less allergenic than raw soybeans^[16]. The IgE binding ability of soybean proteins against the sera of soy-sensitive patients was substantially reduced by *A. oryzae*^[17]. Fermentation of soybeans with *A. oryzae* significantly reduced their IgE binding ability^[18]. The objective of this study was to determine whether *A. oryzae* could reduce the IgE binding ability of egg white.

2 Materials and methods

2.1 Materials

Hen eggs (Lucerne™ premiere large) were purchased from a local supermarket (Edmonton, AB, Canada). *A. oryzae* ATCC 1011 and ATCC 16868 were bought from Microfungus Collection and Herbarium, University of Alberta (Edmonton, AB, Canada). Plasmas from egg allergy patients were obtained from PlasmaLab International (Everett, WA, USA). Ovomucoid was obtained from Neova Technologies (Abbotsford, BC, Canada). Agar, malt extract and peptone were purchased from Bacto™ (Sparks, MD, USA). Coomassie Brilliant Blue R-250, glycine, Laemmli sample buffer, Precision Plus Protein standards, and sodium dodecyl sulfate (SDS) were obtained from Bio-Rad Laboratories (Hercules, CA, USA). Ninhydrin, β -mercaptoethanol, acetone, bovine serum albumin (BSA), cysteine, fluorescein isothiocyanate (FITC), goat-anti-human IgE conjugated with alkaline phosphate, p-nitrophenyl phosphate (pNPP) and Tween-80 were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents used in this research were obtained from Sigma-Aldrich and were of analytical grade.

2.2 Preparation of egg white solutions for fungal treatment

Eggs were wiped with 70% ethanol and flamed to sterilize before breaking, and egg white was separated with egg yolk manually in a sterilized cabinet (Canadian Cabinets, Ottawa, ON, Canada). The pH of the egg white was adjusted to 6.0 by slowly adding 3 mol·L⁻¹ HCl while stirring rapidly.

2.3 Fungal strain preparation

A. oryzae ATCC 1011 and ATCC 16868 were first inoculated from stock cultures into liquid fungal growth medium (pH 5.0–5.5) containing 20 g·L⁻¹ malt extract, 1 g·L⁻¹ peptone and 20 g·L⁻¹ glucose, and incubated at

25°C with gentle shaking (New Brunswick Scientific, Edison, NJ, USA) at 150 r·min⁻¹ for 5 d. Mycelium balls formed naturally during incubation were harvested and washed three times with sterilized 0.85% saline and directly inoculated into acidified egg white (one mycelium aggregate into 20 mL egg white solution). At the same time, some mycelium balls were inoculated onto solid fungal growth medium (20 g·L⁻¹ agar) in flasks, covered with two layers of aluminum foil and grown at 25°C for 5 d. After the agar cultures had sporulated, conidiospores were harvested in 10 mL of sterilized 0.85% saline. The spore suspension was filtered with Whatman Qualitative Filter Paper: Grade 1 Circles (09-805D) and then inoculated into acidified egg white (at a ratio of 2 mL spore suspension and 18 mL egg white) in a 50-mL Erlenmeyer flask. The residual mixture, with spore suspension, was stored at -20°C. After inoculation with mycelium or conidiospores, the Erlenmeyer flask with egg white was covered with Glad plastic wrap and was incubated at 25°C with gentle shaking (orbital shaker, New Brunswick Scientific Innova 4430) with limited oxygen supply. Egg white samples were withdrawn for analysis at 0, 24, 48, 72, 96, and 120 h intervals after inoculation. No visible mycelium was observed in the egg white inoculated with conidiospores over the 120 h incubation period. All samples were prepared in duplicate.

2.4 Determination of amino nitrogen

The amino nitrogen content of egg white sample was determined by the ninhydrin method^[18] following 200-fold dilution in 100 mmol·L⁻¹ sodium phosphate buffer (pH 8.0). After centrifugation (10000 g, 10 min, Eppendorf Centrifuge 5417C, Thermo Fisher Scientific, Waltham, MA, USA), 200 μ L of supernatant was mixed with 100 μ L of ninhydrin color reagent (5 g Na₂HPO₄, 6 g KH₂PO₄, 0.5 g ninhydrin and 0.3 g fructose in 100 mL distilled water, pH 6.7). After incubating in a boiling water bath for 16 min, the mixture was cooled to room temperature for 20 min, and then 500 μ L of dilution solution (0.2% KIO₃ in 40% ethanol) added. The absorbance was determined with a spectrophotometer (Molecular Devices, Sunnyvale, CA, USA) at 570 nm. Glycine was used as the standard for amino nitrogen content calculation.

2.5 Enzyme-linked immunosorbent assay

The IgE binding ability of the *A. oryzae*-treated egg white was analyzed by ELISA^[19] using plasma from four egg-allergic patients (PlasmaLab international, Everett, WA, USA). Egg white samples were diluted to a protein concentration of 5 mg·mL⁻¹ in 50 mmol·L⁻¹ sodium carbonate buffer (pH 9.6, containing 0.6 mol·L⁻¹ NaCl). Then 100 μ L of diluted egg white samples was dispensed into a C96 Maxisorp plate (Nunc Immuno, Thermo Fisher Scientific). The plate was kept at 4°C overnight and

washed four times with 200 μL 0.05 $\text{mol}\cdot\text{L}^{-1}$ Tris-HCl buffer (pH 7.2, containing 0.1% Tween-20) by Skan-Washer 400 (Molecular Devices). To achieve blocking, 200 μL of 1 $\text{mol}\cdot\text{L}^{-1}$ PBS buffer containing 2% BSA (pH 7.4) was added to the unoccupied space of wells and kept at room temperature for 1 h. After washing using the same washing buffer above, 100 μL diluted human plasma (1:100, v/v, in 1 $\text{mol}\cdot\text{L}^{-1}$ PBS containing 1% BSA) was added to each well and the plate incubated with shaking (150 $\text{r}\cdot\text{min}^{-1}$) at 37°C for 2 h, followed by four washes. Then 100 μL of diluted goat-anti-human IgE conjugated with alkaline phosphate (diluted 1000 times in 0.05 $\text{mol}\cdot\text{L}^{-1}$ Tris-HCl buffer, pH 8.0, containing 1% BSA, 0.1% Tween-20 and 1 $\text{mmol}\cdot\text{L}^{-1}$ MgCl_2) was added to each well, and the plate incubated at room temperature for 2 h with shaking. The plate was then washed five times to remove all the remaining unbound antibodies, and the bound antibodies were detected by adding 100 μL of p-nitrophenyl phosphate (pNPP) solution, a color reagent. After 30 min of incubation, 100 μL of 3 $\text{mol}\cdot\text{L}^{-1}$ NaOH was added to stop the color reaction, and the plate was read with a spectrophotometer (Molecular Devices, Sunnyvale, California) at 405 nm wavelengths. Each sample was applied to eight wells for analysis and the highest and lowest absorbance values for each sample were discarded.

2.6 SDS-PAGE analysis of egg white proteins

Egg white proteins were analyzed using SDS-PAGE^[20] using samples diluted with 100 $\text{mmol}\cdot\text{L}^{-1}$ Tri-HCl, pH 8, containing 1% (w/v) SDS to a concentration of 2 $\text{mg}\cdot\text{mL}^{-1}$. After adding 45 μL of Laemmli sample buffer and 5 μL of β -mercaptoethanol to 50 μL diluted egg white, the mixture was heated at 95°C for 15 min with gentle shaking by Eppendorf Thermomixer R (Thermo Fisher Scientific). After centrifugation at 14000 g for 5 min, 25 μL sample supernatant was loaded to 12% SDS-PAGE ready gel (Bio-Rad Laboratories) and Precision Plus Protein standards with a molecular weight range of 10 to 250 kDa were also loaded. The gel was run at 200 V and stained in methanol/acetic acid/water (5:1:4) (v/v/v) containing 1/500 (w/v) Coomassie Brilliant Blue R-250 for 2 h, destained by five washes of 15 min in methanol/acetic acid/water (5:1:4) (v/v/v), and then washed in deionized water. The gels were scanned using a gel scanner (Alpha Innotech Corp., San Leandro, CA, USA) with FluorChem SP software.

2.7 SDS-PAGE analysis of egg white containing FITC labeled ovomucoid

Ovomucoid was labeled and purified according to the manufacturer's manual of Fluoro Tag™ FITC Conjugation Kit (Sigma-Aldrich). Ovomucoid (5 $\text{mg}\cdot\text{mL}^{-1}$) was mixed with FITC (1 $\text{mg}\cdot\text{mL}^{-1}$), prepared in 0.1 $\text{mol}\cdot\text{L}^{-1}$ sodium carbonate-bicarbonate buffer (pH 9.0), at a ratio of 4:1

(v/v). The mixture was covered with aluminum foil to protect the sample from light and incubated at room temperature for 2 h with gentle shaking. Labeled ovomucoid was purified by Sephadex G-25M column (9.1 mL bed volume, 5 cm bed height and 1.5 mL maximal sample volume) and eluted by 10 $\text{mmol}\cdot\text{L}^{-1}$ PBS (pH 7.4). Eluted fractions were measured at absorbance of 280 and 495 nm by spectrophotometer (NanoDrop 2000C, Thermo Fisher Scientific) to select the fractions that contain the ovomucoid conjugated with FITC.

Growth of *A. oryzae* mycelium in acidified egg white supplemented with labeled ovomucoid was performed as described above but on a small scale. Egg white solution, pH 6.0, was mixed with purified FITC labeled ovomucoid in a ratio of 9:1. The washed *A. oryzae* mycelium was inoculated into the mixtures and, after incubation for 48 h covered with aluminum foil with gentle shaking, samples were taken for SDS-PAGE analysis as described above and the fluorescence of FITC was read with a Typhoon Variable Mode Imager (GE Life Sciences, Sunnyvale, CA, USA).

2.8 Matrix-assisted laser desorption/ionization time of flight mass spectrometry analysis of *A. oryzae*-treated egg white proteins

MALDI-TOF-MS was used to determine the molecular weights of egg white proteins before and after treatment. Samples were prepared according to the procedure of a two-layer method^[21]. The first thin matrix layer was formed by loading 0.7 μL of 10 $\text{mg}\cdot\text{mL}^{-1}$ 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) in 4:1 acetone/methanol (HPLC grade) (v/v) onto a clean MALDI target. The matrix layer was spread and dried immediately after loading. Then 2 μL of diluted egg white sample was mixed with 2 μL of saturated sinapinic acid in 1:1 acetonitrile/water (v/v) and 1 μL of this mixture applied onto the first layer of the matrix. After drying, the sample was desalted by adding 5 μL of water onto top of the dry spot and the liquid blown off by an air pulse after 10 s. This was repeated five times. Each sample was applied onto six spots, and at least three spots of each sample were analyzed. MALDI analysis was carried out on an Applied BioSystems Voyager Elite MALDI (Foster City, CA, USA) time of flight mass spectrometer in a positive linear ion mode.

2.9 In-gel digestion of ovomucoid

A. oryzae-treated and acidified egg whites were digested in-gel^[22]. Ovomucoid bands were excised from the SDS-PAGE gel, transferred into low-retention microcentrifuge tubes (Thermo Fisher Scientific) and destained by double incubation with 200 μL of 200 $\text{mmol}\cdot\text{L}^{-1}$ ammonium bicarbonate with 40% acetonitrile for 30 min followed by

washing two times with 200 μL acetonitrile. After drying, the gel bands were reduced with 40 μL 10 $\text{mmol}\cdot\text{L}^{-1}$ dithiothreitol for 30 min, followed by alkylation with 40 μL 50 $\text{mmol}\cdot\text{L}^{-1}$ iodoacetamide for 30 min in the dark. After removing iodoacetamide, the gels were washed with 100 $\text{mmol}\cdot\text{L}^{-1}$ ammonium bicarbonate and acetonitrile, and dried in an Eppendorf Vacofuge (Thermo Fisher Scientific). Dried gel pieces were digested overnight at 37°C with 0.8 μg modified trypsin (Promega, Madison, WI, USA) in 50 $\text{mmol}\cdot\text{L}^{-1}$ ammonium bicarbonate. After digestion, peptides were extracted into new microcentrifuge tubes with 30 μL of 100 $\text{mmol}\cdot\text{L}^{-1}$ ammonium bicarbonate followed by extracting two times with 30 μL solution containing 5% formic acid and 50% acetonitrile in water. The resulting solution was partially evaporated using an Eppendorf Vacofuge to ~ 15 μL .

2.10 LC-ESI MS and MS/MS analysis

The in-gel tryptic digests were subjected to LC-ESI MS and MS/MS analysis using hybrid quadrupole orthogonal acceleration time of flight QToF Premier (Waters Corp., Milford, MA, USA) online connected with the Waters nanoACQUITY ultra high performance liquid chromatography (UPLC) system. Briefly, 4 μL of the ovomucoid digest containing peptides and glycopeptides were loaded onto a micro precolumn C_{18} cartridge that was connected to a 75- μm (internal diameter) by 150-mm Atlantis dC₁₈ column (Waters Corporation). After a 2-min trap wash in the precolumn with solvent A (1% acetonitrile in water containing 0.1% formic acid) at a flow rate of 10 $\mu\text{L}\cdot\text{min}^{-1}$, the digest was separated with a gradient of 1%–65% solvent B (acetonitrile, 0.1% formic acid) over 35 min at a flow rate of 300 $\text{nL}\cdot\text{min}^{-1}$. The column was connected to a QToF premier (Waters Corporation) for ESI-MS and MS/MS analysis of the effluent. The collision energy used to perform MS/MS was varied according to the mass and charge state of the eluting ions. The data acquisition was conducted using MassLynx (Waters Corporation; MassLynx, version 4.1).

2.11 Statistical analysis

All data was analyzed by one-way analysis of variance using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA). The significant differences were determined using Dunnett's test at $P < 0.05$ ^[23].

3 Results and discussion

3.1 IgE binding ability of *A. oryzae*-treated egg whites

Effect of incubation time on the IgE binding ability of egg white proteins by *A. oryzae* is shown in Fig. 1. The higher the absorbance in the ELISA test, the higher the IgE

binding ability of egg white samples. The IgE binding ability of egg white proteins was significantly reduced within the first 24 h of incubation. When inoculated with conidiospores, the IgE binding ability of egg white proteins was decreased to a lesser extent. The results show that mycelium of *A. oryzae* is more effective than conidiospores in reducing IgE binding ability of egg whites.

3.2 Amino nitrogen content and protein profile analysis

A. oryzae secretes proteases and deglycosylating enzymes, which determine the texture and the flavor of many fermented food products^[24]. When acidified egg white was inoculated with conidiospores and mycelium of *A. oryzae* ATCC 1011 and ATCC 16868, the total free amino nitrogen content did not change over 120 h of incubation (data not shown), which indicated that there was no significant protein degradation occurring during incubation. SDS-PAGE analysis also revealed that the four major egg white allergens were not altered during the 120 h of incubation (data not shown). As the IgE binding ability of egg white proteins was almost eliminated by incubation with *A. oryzae*, FITC labeled ovomucoid was incubated with egg white to determine if this dominant egg allergen was degraded. The fluorescence intensity of ovomucoid band was not affected during incubation, compared to the non-incubated control, and there were no additional bands generated during incubation (data not shown). This result suggests that there was no cleavage of FITC adjacent moieties in ovomucoid, which differed from previous observation in *Lactobacillus delbrueckii*-treated egg whites^[25]. In addition, no obvious migration of fluorescence bands was observed after incubation, which indicates that the labeled ovomucoid was not substantially degraded by *A. oryzae*.

3.3 MALDI-TOF-MS analysis of *A. oryzae*-treated egg white proteins

The effect of *A. oryzae* treatment on the molecular weight of egg white proteins analyzed by MALDI-TOF-MS is shown in Fig. 2. According to the molecular weight of egg white proteins, peaks 1 to 5 represents lysozyme, ovomucoid, ovotransferrin (doubly charged), ovalbumin and ovotransferrin (singly charged), respectively. No significant protein degradation was observed in treated egg whites, confirming observations with SDS-PAGE. However, peak 2 shifted from 28973 to 27271 and 27582 Da with ATCC 1011 and ATCC 16868, respectively, and the main peaks domain shifted from 25490–29970 to 25130–29770 Da and 25150–29725 Da, respectively. These results suggest that incubation of egg white with mycelium of *A. oryzae* ATCC 1011 and ATCC 16868 modifies ovomucoid.

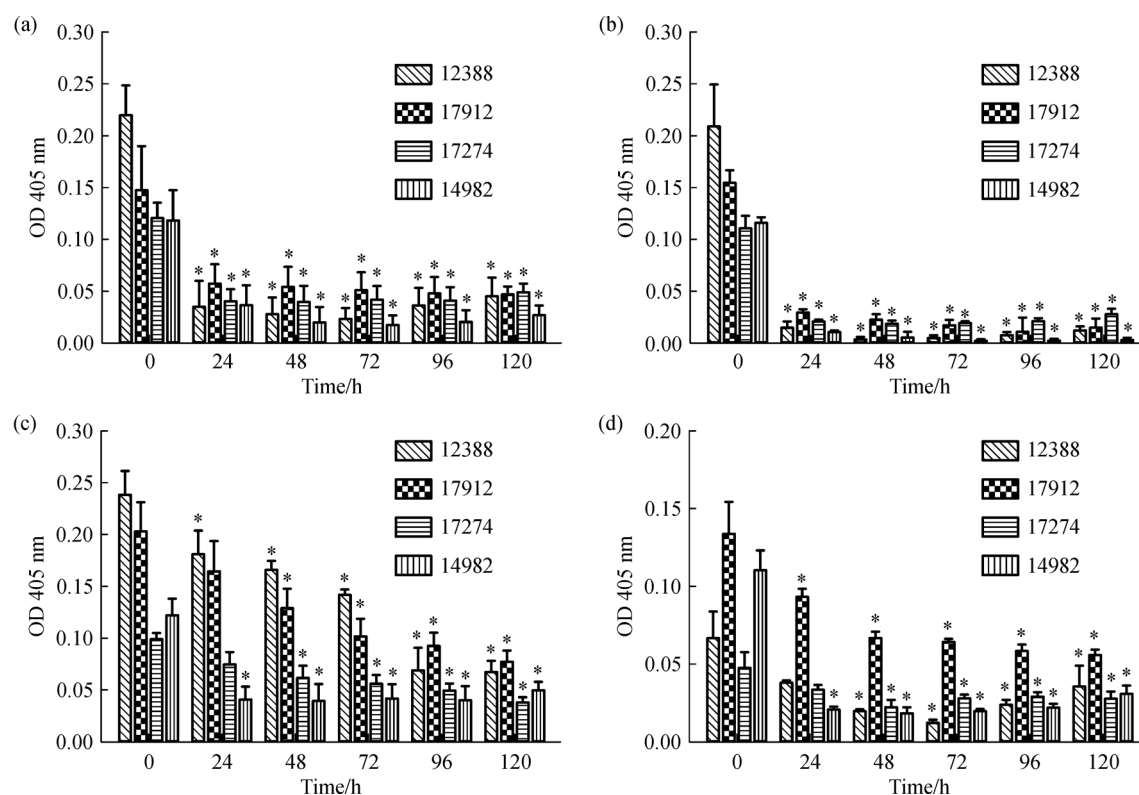


Fig. 1 Effect of incubation time on the IgE binding ability of egg white proteins incubated with mycelium and conidiospores of *A. oryzae* ATCC 16868 and ATCC 1011; mycelium ATCC 1011 (a), ATCC 16868 (b), conidiospores ATCC 1011 (c), and ATCC 16868 (d). Results are means±standard deviation of triplicate analyses each of two independent experiments using plasma from four egg allergy patients (12388, 17912, 17274 and 14982). The statistical analysis by Dunnett's test of one way ANOVA indicated that the IgE binding ability of egg white incubated with mycelium of ATCC 1011 and ATCC 16868 showed significant differences ($P < 0.001$, marked with asterisk) at 24, 48, 72, and 96 h of incubation for all plasma samples from egg-allergy patients.

3.4 LC-ESI MS and MS/MS analysis

To investigate the specific modification of ovomucoid during incubation, ovomucoid bands from SDS-PAGE of control and *A. oryzae*-treated egg whites were excised, digested with trypsin and subjected to nano LC-ESI MS and MS/MS analysis. The data was submitted for a database search by Mascot (Matrix Science website) to further examine possible protein degradation of ovomucoid. Identified tryptic peptides of ovomucoid in the control and treated samples are presented in Table 1. The N-terminal part of the ATCC-16868-treated sample (AEVDCSR) was not identified by Mascot, while the C-terminal part was found in both samples. This result suggests that a degradation of a few amino acids at the N-terminus of ovomucoid occurred during incubation with *A. oryzae* ATCC 16868.

It is unlikely, however, that a degradation of only this short fragment would result in the mass change of ~1700 Da detected by MALDI-TOF-MS. Therefore, we examined the possibility of glycan degradation. Glycosyl hydrolases of *A. oryzae*^[26] can remove carbohydrate moieties in egg white proteins. Many egg proteins are

glycoproteins and the dominant egg allergen, ovomucoid, also contains 25% to 30% carbohydrate moiety. To determine whether degradation of the carbohydrate moiety of ovomucoid occurred, we compared the presence of tryptic glycopeptides in the control and *A. oryzae*-treated egg whites. The glycopeptides were eluted between 13 and 23 min. All the glycopeptides belonged to various peptides containing a glycosylation site on N10. Figure 3 shows a representative MS spectrum of glycopeptides that eluted between 17 and 21 min in the control and *A. oryzae*-treated samples, the majority of which belong to a peptide FPNATDKEGKDVLC*^{NK}. The composition of the glycopeptides was assessed from the MS/MS spectrum when available and there were differences in masses on the MS spectrum. A summary of the glycan compositions is presented in Table 2. The MSMS of a quadruply-charged glycopeptide FPNATDKEGKDVLC*^{NK} + Core + 6 HexNAc at m/z of 1012.1 is shown in Fig. 4 as an example. By comparing the glycopeptides with the peptide FPNATDKEGKDVLC*^{NK}, it appears that there were no changes in the glycan composition resulting from the treatment, as there were no differences in the MS spectrums.

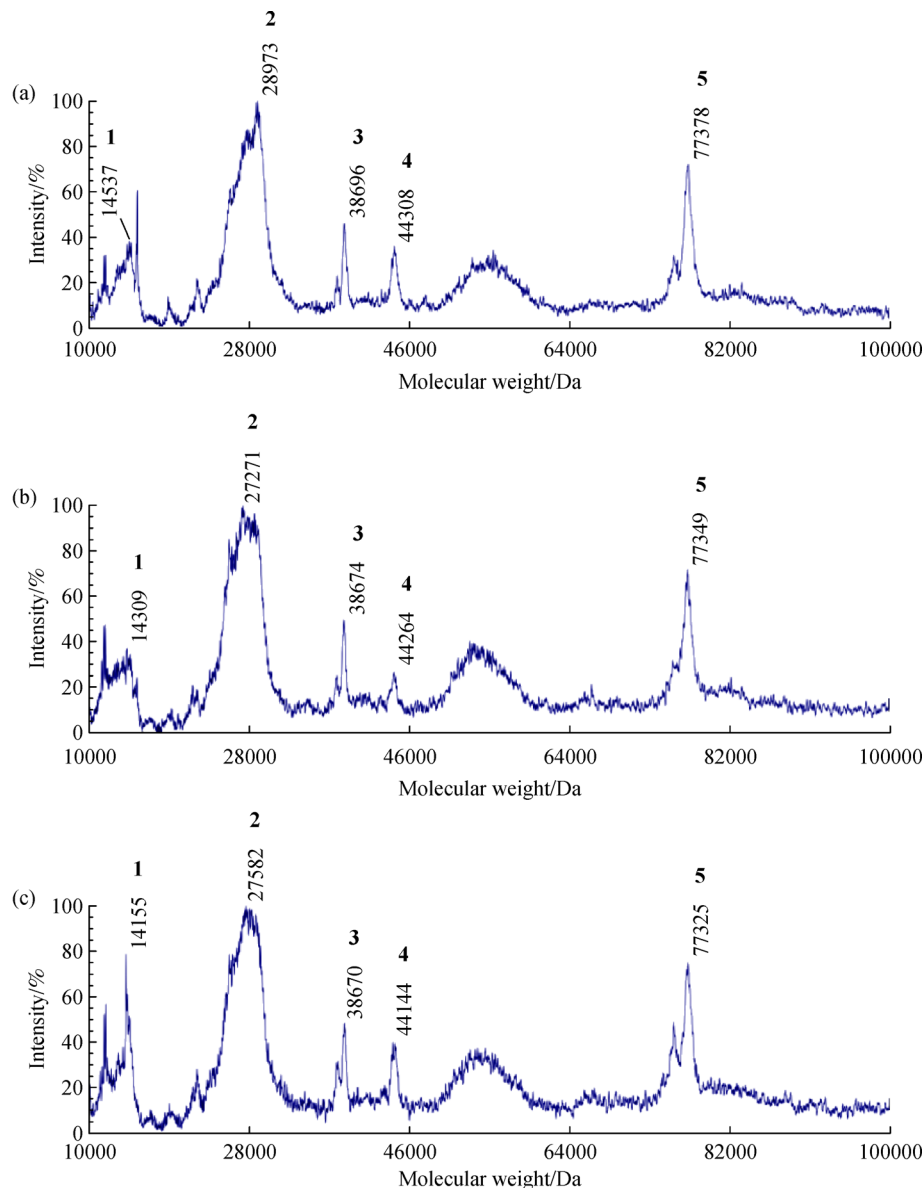


Fig. 2 MADLI-TOF-MS analysis of egg white proteins after incubation with *A. oryzae* ATCC 1011 or ATCC 16868. Acid egg white with (a) and without incubation with the mycelium of ATCC 1011 (b) or ATCC 16868 (c) were analyzed. Peaks 1–5 represent lysozyme, ovomucoid, ovotransferrin (doubly charged), ovalbumin and ovotransferrin, respectively.

Although there was no evidence of glycan degradation, it is possible that glycan degradation was not detected due to the natural glycan heterogeneity and that other glycosylation sites were affected. Compared to the other major egg white proteins, ovomucoid is more resistant to hydrolysis because of its primary sequence and conformational structure. Although ovomucoid contains no inter-domain disulfide bonds, each domain is cross-linked by three intra-domain disulfide bonds, rendering ovomucoid resistance to heat denaturation and digestive enzymes^[5,6]. In a further experiment (data not shown), we also inoculated the mycelium of *A. oryzae* into egg white at pH 9.0. However, no reduction of egg white IgE binding ability was observed in these trials. The pH of the egg

white could affect either the production or activity of enzymes of *A. oryzae*. It is evident that pH 6.0 is more conducive to the reduction IgE binding ability of egg white incubated with *A. oryzae*, and this is in accordance with the findings of Ferea et al.^[27] that more protease genes of *A. oryzae* function under acidic pH.

4 Conclusions

A. oryzae is widely used for preparing many traditional Asian foods due to its high hydrolytic activity. The possibility of using *A. oryzae* in reducing egg IgE binding property was tested in this study. The results show the IgE

Table 1 Tryptic peptides of ovomucoid found by Mascot in egg white samples incubated with *A. oryzae* ATCC 1011 and ATCC 16868

| Start-End ^a | Observed (m/z) | Observed molecular weight/Da | Sequence | Acidic egg white | ATCC 1011 | ATCC 16868 |
|------------------------|---|------------------------------|---------------------------------------|------------------|-----------|------------|
| 25-41 | 481.7 ^(+ 4) 642.0 ^(+ 3) | 1922.9 | AEVDCSRFPNATDKEGK | - | V | - |
| 25-48 | 688.8 ^(+ 4) | 2751.3 | AEVDCSRFPNATDKEGKDVLVCNK | V | - | |
| 32-41 | 553.8 ^(+ 2) | 1105.6 | FPNATDKEGK | - | - | V |
| 32-48 | 645.7 ^(+ 3) 484.5 ^(+ 4) | 1933.9 | FPNATDKEGKDVLVCNK | V | V | - |
| 81-87 | 437.7 ^(+ 2) | 873.3 | EHDGECK | - | - | V |
| 107-113 | 446.2 ^(+ 2) 454.2 ^(+ 2) | 890.4 906.4 | VMVLCNR VMVLCNR (oxidation) | - | V | V |
| 114-136 | 881.0 ^(+ 3) 1321.0 ^(+ 2) 661.0 ^(+ 4) | 2640.1 | AFNPVCGTDGVTYDNECLCAHK | V | V | V |
| 114-146 | 928.4 ^(+ 4) 1237.6 ^(+ 3) | 3709.7 | AFNPVCGTDGVTYDNECLCAHK- VEQGASVDKR | V | V | - |
| 137-146 | 363.5 ^(+ 3) 544.8 ^(+ 2) | 1087.6 | VEQGASVDKR | - | V | V |
| 154-183 | 1128.5 ^(+ 3) 846.6 ^(+ 4) | 3382.4 | ELAAVSVDCEYPKPDG- TAEDRPLCGSDNK | V | V | V |
| 184-188 | 582.3 | 581.3 | TYGNK | - | - | V |
| 189-209 | 785.7 ^(+ 3) 1178.0 ^(+ 2) | 2354.1 | CNFCNAVVESNGTLTSLSHFGK | - | V | V |

Note: ^a The start and end sequences are based on the ovomucoid sequence in UniProt database (P01005). Sequence 1-24 is a signal peptide; therefore the protein starts at AA25.

Table 2 Glycan composition of ovomucoid glycopeptides of a peptide FPNATDKEGKDVLVC*NK (mass of 1935.0 Da) that contain a glycosylation site on N10

| No. | Observed (m/z) | Charge | Mass/Da | Glycan composition |
|-----|----------------|--------|---------|-----------------------|
| 1 | 943.1 | 3 | 2827.3 | Core |
| 2 | 1078.5 | 3 | 3233.5 | Core + 2HexNAc |
| 3 | 1146.2 | 3 | 3436.6 | Core + 3HexNAc |
| 4 | 1200.2 | 3 | 3598.6 | Core + 3HexNAc + Hex |
| 5 | 1213.9 | 3 | 3639.7 | Core + 4HexNAc |
| 6 | 1267.9 | 3 | 3801.7 | Core + 4HexNAc + Hex |
| 7 | 1281.6 | 3 | 3842.8 | Core + 5HexNAc |
| 8 | 1335.6 | 3 | 4004.8 | Core + 5HexNAc + Hex |
| 9 | 1349.3 | 3 | 4045.9 | Core + 6HexNAc |
| 10 | 1012.2 | 4 | 4045.9 | Core + 6HexNAc |
| 11 | 1403.3 | 3 | 4207.9 | Core + 6HexNAc + Hex |
| 12 | 1457.3 | 3 | 4369.9 | Core + 6HexNAc + 2Hex |

Note: The glycopeptides eluted between 17 to 21 min. All the glycopeptides were found in control samples and those treated with *A. oryzae* ATCC 1011 and ATCC 16868, as can be seen in Fig. 4. HexNAc, N-acetylhexosamine; Hex, hexose; core, chitobiose core of two N-acetylglucosamines and three mannoses. Glycopeptide mass is for singly charged state.

binding property of egg white was significantly reduced after incubation with *A. oryzae*. However, characterization of treated egg white protein samples did not reveal major protein degradation. Nevertheless, a loss of around 1700 Da mass was found in ovomucoid by MALDI-TOF-MS after incubation with *A. oryzae* ATCC 16868 and a

possible loss of the N terminus was indicated by LC-MS/MS analysis. Ovomucoid contains about 25% to 30% of glycan, but no obvious glycan degradation was found during the treatment. Our study suggests that slight structural modification of ovomucoid could lead to significant IgE binding ability reduction of whole egg white.

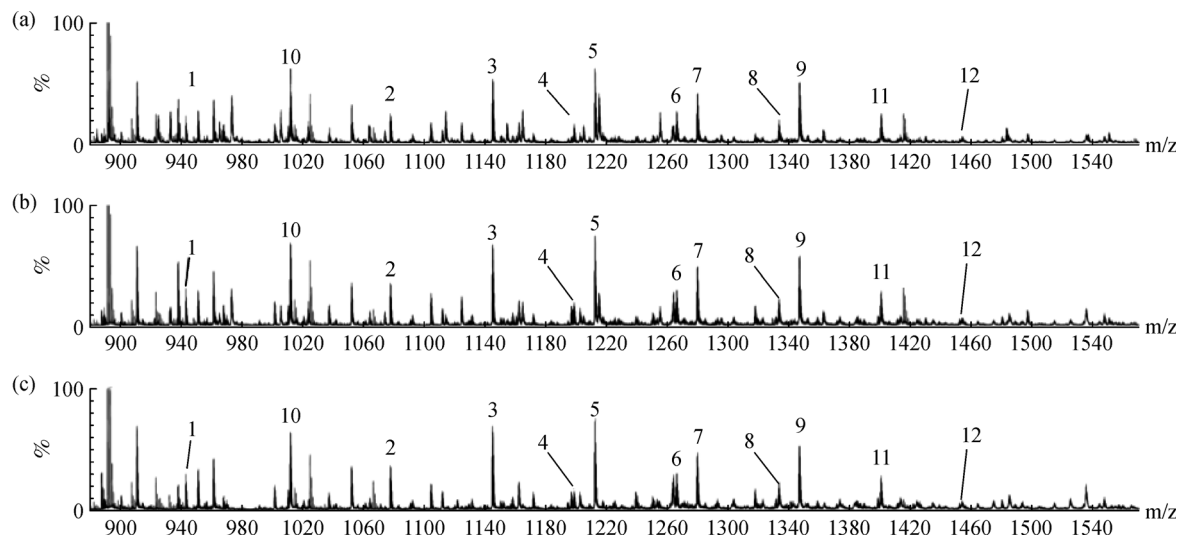


Fig. 3 MS spectrum of peaks eluted between 17 and 21 min of control and treated eggs whites; control (a), incubated with *A. oryzae* ATCC 1011 (b) and ATCC 16868 (c). The mass range focuses on glycopeptides that correspond to a peptide FPNATDKEGKDLVC*NK with glycosylation site N10. Identified glycans are numbered 1–12. The composition of the glycans can be seen in Table 2. No differences in the glycan composition or intensity was observed between the control and *A. oryzae*-treated samples.

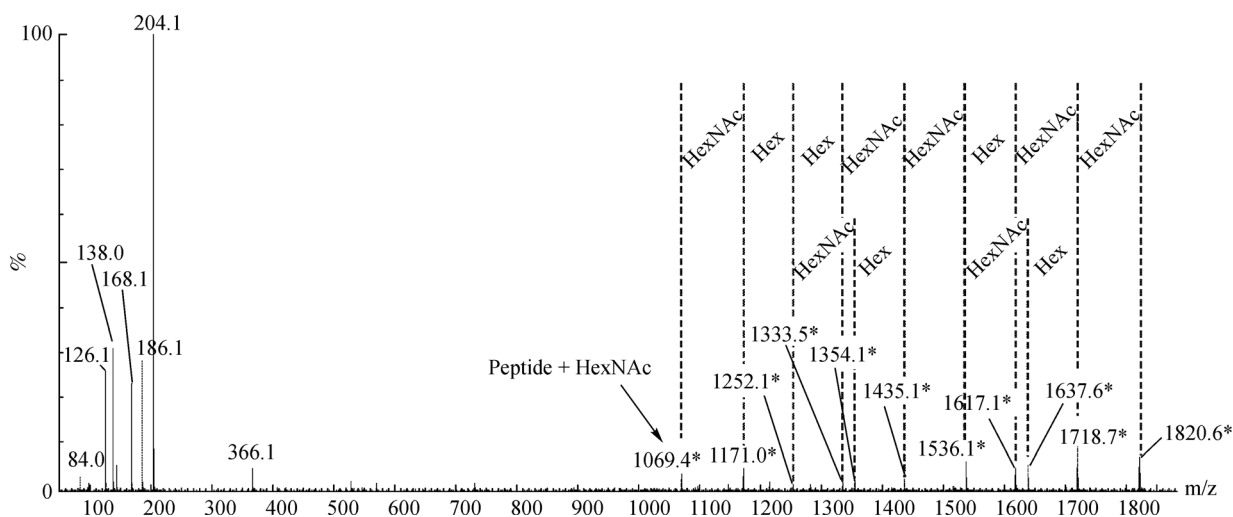


Fig. 4 MS/MS spectrum of a glycopeptide FPNATDKEGKDLVC*NK (mass of 1935.0 Da) with a glycan chain that is composed of the N-glycan core (two N-acetylglucosamines and three mannoses) and six additional N-acetylglucosamines on the core. The MS/MS spectrum of the quadruply-charged glycopeptide with m/z of 1012.2 shows a strong peak at 204.1 and at 366.1—an indication of a glycopeptide (HexNAc and HexNAc + Hex, respectively). Ions marked with an asterisk are doubly-charged. The presence of a doubly-charged ion at 1354.1 indicates a bisected glycan structure that is typical for ovomucoid.

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Compliance with ethics guidelines Sen Li, Marina Offengenden, Michael G. Gänzle, and Jianping Wu declare that they have no conflicts of interest or financial conflicts to disclose.

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