

Polyphenol-Oxidase-Catalyzed Cross-linking of Ara h 2: Reaction Sites and Effect on Structure and Allergenicity

Running title: PPO-Catalyzed Cross-linking of Ara h 2

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Abstract

Background: Peanut is among the most common of food allergies, and one of its allergens is Ara h 2. A previous study revealed that this allergen was recognized by serum Ig E in over 90% of a peanut-allergic patient population. Enzymatic cross-linking is a popular processing method used to tailor food functionality, such as antigenicity.

Result: The cross-linking reactions of Ara h 2 were catalyzed by polyphenol oxidase (PPO), and the relevant reaction sites were identified using mass spectrometry and StavroX software. Two pairs of intramolecular cross-linking peptides and two intermolecular cross-linking peptides were found. Intramolecular cross-linking was speculated to occur between ARG¹³¹ (aa 116-131) and TYR⁶⁵ (aa 63-80) and between TYR⁶⁰ (aa 56-62) and ARG⁹² (aa 92-102); the intermolecular

cross-linking sites were ARG³¹, TYR⁸⁴, TYR⁸⁹ and TYR⁶⁵, TYR⁷², ARG⁹², and ARG¹⁰². Three out of four cross-linking peptides were found in alpha-helices, and destruction of this secondary structure caused a loose tertiary structure. Although seven linear allergen epitopes were involved in cross-linking, the Ig E binding capacity of protein changed slightly, while its sensitization potential decreased in mouse model.

Conclusion: Exploring the structural change of Ara h 2 after cross-linking is beneficial to further understand the influence of structure on sensitization. This result indicated the future possibility of precision processing on structure of proteins to improve their properties.

Keywords: Ara h 2; cross-linking; polyphenol oxidase; structure; allergenicity;

INTRODUCTION

Peanut (*Arachis hypogaea*) is one of the most common food allergies; the symptoms of an allergic reaction to this food may include vomiting, abdominal pain, angioedema, nausea, loss of consciousness, and even death^{1, 2}. Patients must maintain strict avoidance because allergic individuals can immediately exhibit adverse reactions upon accidental ingestion of a trace of peanut³. However, this practice is very difficult because of the extensive use of peanut and peanut products in foods⁴.

Peanut proteins include albumins and the storage proteins arachin and conarachin, which comprise about 87% of the total peanut protein content⁵. Several peanut proteins belonging to different families of seed storage proteins have been identified to be allergenic⁶. Among these proteins, Ara h 2 was considered to be a major allergen and recognized by serum Ig E in over 90% of a peanut-allergic patient population^{7, 8}.

Processes altering protein properties by structural improvement have been developed⁹. Enzymatic cross-linking, for example, is a popular processing method to tailor food functionality. After cross-linking, the 3D structure, surface characteristics of the molecule, ligand binding, Ig E

binding, and antigenicity of a protein has changed^{10, 11}. Polyphenol oxidase (PPO) is a copper-containing enzyme existing extensively in fruits and vegetables that can induce protein cross-linking^{12, 13}. The protein could form a quinone derivative by catalytic oxidation of the phenol group in tyrosine residues, which then reacted with the amino, sulfhydryl, or tryptophan residue of another protein¹⁴⁻¹⁶. As two out of the three main linear epitopes of Ara h 2 contain tyrosine, altering the structure of these epitopes by cross-linking may lead to decreasing allergenicity^{17, 18}.

Our preview study showed that Ara h 2 could cross-link catalyzed by PPO, and cross-linking reaction could change digestibility and potential allergenicity of Ara h 2²¹. In the present work, the intramolecular cross-linking was proved, and the reaction sites of Ara h 2 were identified. The effect of reaction on spatial structure was discovered, which could help to explain the mechanism of change on the potential allergenicity.

Materials and methods

Materials

Chemicals

DEAE-Sepharose[®] Fast Flow Resin (16 mm×250 mm) was

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purchased from Amersham Biosciences (Freiburg, Germany). The 96-well microtiter plate was purchased from Corning (Midland, MI, USA). Complete Freund's adjuvant, incomplete Freund's adjuvant, bovine serum albumin (BSA), the sources of electrophoresis chemicals, goat anti-mice Ig G-peroxidase, goat anti-mice Ig G1-peroxidase, goat anti-human Ig E-peroxidase, Tween 20 were obtained from Sigma–Aldrich (St. Louis, MO, USA). IL-4, IL-5, IL-13, and IFN- γ were assayed using ELISA kits (Ebioscience, San Diego, USA) according to the instructions of the manufacturer. Pre-stained protein ladder was purchased from MBI Fermentas (Burlington, Canada).

Human sera

The subjects ($n = 8$) gave informed consent and assent. Subjects with a strong history of severe systemic reactions to peanuts who had physical examination and objective clinical manifestations. Specific-peanut Ig E of at least $2.0 \text{ IU} \cdot \text{mL}^{-1}$ were recruited in our study. A pooled serum sample (serum pool) containing sera from the eight patients on the basis of the content of anti-peanut Ig E was prepared and frozen at -80°C until used. The specific Ig E level of serum pool was $5.2 \text{ kU} \cdot \text{L}^{-1}$ (as confirmed by Immuno CAP 100, Phadia, Uppsala, Sweden).

Ara h 2 and PPO

Ara h 2 was purified by anion exchange chromatography from crude peanut extract in Tris–HCl buffer (pH 7.2) according to the method of Hu *et al*¹⁹. The PPO was isolated from mushroom (*Agaricus bisporus*) after acetone degreasing in 50 mmol·L⁻¹ phosphate buffer at 4°C in accordance with previous reports²⁰.

Methods

Cross-linking condition

Ara h 2 (2 mg·mL⁻¹) was dissolved in a 50 mmol·L⁻¹ solution of sodium phosphate (pH 7.0) and PPO was dissolved in a 50 mmol·L⁻¹ solution of sodium phosphate. The prepared protein solution and PPO solution were dispersed in 50 mmol·L⁻¹ solution of sodium phosphate and incubated for 80 min as previously described²¹. The reaction was stopped by heating the samples (80°C, 5 min), then the samples were stored at -20°C until use.

Tricine-SDS-PAGE and degree of cross-linking

Tricine–SDS-PAGE was performed as previously described to separate proteins in the range from 1 to 100 kDa^{22, 23}. Protein samples were reduced and denatured in reducing sample buffer (1:1 v/v).

Electrophoresis (16.5% separating gel, 10% spacer and 4% stacking gel) was performed at a constant voltage of 30 V in 4% sample (stacking) gel for 1 h and 100 V in the separating gel for 2-2.5 h.

The band at 17 kDa was excised for mass spectrometry (MS) analysis as monomer Ara h 2, which might include native Ara h 2 and intramolecular cross-linked Ara h 2. The 34 kDa peptide and above band were polymerized Ara h 2 which were excised as intermolecular cross-linked sample.

Liquid chromatography (LC)–MS and data analyses

The cross-linking samples were analyzed following the work of Hoopmann *et al*²⁴. After digesting the complex with trypsin (1:100 ratio, 37°C overnight), the peptides were analyzed using reverse-phase liquid chromatography and MS at high resolution using a Q-Exactive mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). In order to exclude the ions previously targeted a second injection was made after initial run in the MS/MS analysis. The resulting peptides' MS/MS spectra were analyzed using StavroX to identify the cross-linked peptides.

Circular dichroism analysis

Ara h 2 catalyzed by PPO were analyzed by circular dichroism.

Circular dichroism (CD) spectroscopy was performed using a JASCO J810 spectropolarimeter (JASCO, Groß-Umstadt, Germany) on 0.15 mg·mL⁻¹ protein solutions¹⁹. The contents of different secondary structures were calculated using secondary structure software (<http://dichroweb.cryst.bbk.ac.uk/html/home.shtml>).

UV absorption spectral analysis

UV absorption spectral analysis was performed using a UV WinLab V6 spectrophotometer (PerkinElmer, Japan). The concentration of detected protein was 0.3 mg·mL⁻¹. The UV absorption spectra were scanned from 250 to 350 nm at a rate of 480 nm·min⁻¹.

Spatial structure of Ara h 2

The spatial structure of a protein is widely used to analyze the spatial location of amino acid residues and peptides. The spatial structure of Ara h 2 was built on the SWISS MODEL website (<http://swissmodel.expasy.org/>) using Ara h 2 (PDB database Number 3ob4.1.A) as a model^{25, 26}. Then the cross-linking peptides were marked and shown by PyMOL (version 1.0.2) software.

Ig E binding of cross-linked Ara h 2 products

Ig E binding experiment was performed by competitive ELISA as

described previously²⁷. In short, microplate A was pre-coated with purified native Ara h 2 ($3 \mu\text{g}\cdot\text{mL}^{-1}$) overnight and washed in the second day. Microplate B was blocked with 5% non-fat milk (w/v) (1 h, 37°C), cross-linking Ara h 2 and anti-Ara h 2 Ig E polyclonal antibodies were added in order and incubated 100 μL of goat anti-human Ig E conjugated with horseradish peroxidase was added for 1 h. After color development, the reaction was terminated by adding H_2SO_4 .

In vivo allergenicity assessment

The animal experiment was performed on female Balb/c mice (6 weeks of age) from the Institute of Occupational Medicine of Jiangxi (permission number SYXK (Gan) 2009-003)^{28, 29}. The mice were maintained in cages at a temperature of $23 \pm 2^\circ\text{C}$, relative humidity of 50-55%, and 12 h light/dark cycle. Water and food pellets were provided ad libitum. All animal experimental procedures were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of the People's Republic of China.

Oral sensitization to peanut was prepared as previously described Wu *et al*³⁰. All 27 mice were divided in three groups. The first group or,

the control group was exposed to 10 µg of cholera toxin only. The other two groups were exposed to 10 µg of cholera toxin and 1 mg of protein (native Ara h 2 for group 2, Ara h 2 catalyzed by PPO for group 3). An intragastric gavage of same dosage was repeated once a week for 5 weeks. On day 42, all mice received a challenge of 2 mg of peanut proteins intragastrically. To measure the level of specific antibodies, sIg G and sIg G1 specific to Ara h 2, tail blood was collected 30 min after the oral challenge on day 42 and made into serum. The sIg G and sIg G1 level were performed via indirect ELISA. Mice were sacrificed by vertebral dislocation, and spleens were harvested under sterile conditions for cell culture.

Cell culture and cytokine measurements

Cell cultures were performed as previously described^{30, 31}. Briefly, spleen single-cell suspensions (3.75×10^5 cell per mL cells in 400 µL of complete RPMI 1640) were incubated with peanut protein ($20 \mu\text{g} \cdot \text{mL}^{-1}$) or without peanut protein as a negative control for 72 h. After centrifugation for 5 min at 1000 g, the supernatant was collected and stored at -80°C until analysis. IL-4, IL13, and IFN-γ were assayed using ELISA kits according to the instructions of the manufacturer.

Statistical analysis

All data were processed in Origin (version 6.0) and reported as the average and standard deviation, unless otherwise stated. The bivariate Pearson correlation was analyzed in SPSS (Chicago, IL, USA). Differences were considered significant and very significant when the p-values were less than 0.05 and 0.01, respectively.

RESULTS

Cross-linking reaction

While the molecular weight of native Ara h 2 was approximately 17 KDa in lane 1, the dimers and polymers of cross-linked Ara h 2 could be found in lane 2 (Fig. 1). The band at approximately 34 kDa might indicate the dimers of Ara h 2, and the bands above 100 kDa might be polymers of the protein. This result proved that PPO catalyzed intermolecular cross-linking reactions in Ara h 2²¹. The band at approximately 17 KDa in lane 2 believed to contain native Ara h 2 and some intramolecular cross-linked proteins. The band at 17 kDa was excised as a monomer sample, while dimers and polymers were applied as intermolecular cross-linked samples.

Cross-linking sites

The protein samples were analyzed using LC–MS and StavroX software to identify cross-linked peptides (Fig. 2A). Peptide 1 was found to be cross-linked to peptide b and peptide 2 was found to be cross-linked to peptide c in the intramolecular cross-linked samples. In intermolecular cross-linked samples, cross-linking was observed between peptide 2 and peptide b and between peptide 3 and peptide a. Cross-linking peptides overlapped in 7 out of 10 linear allergen epitopes of Ara h 2.02³², as marked in the protein sequence in Fig. 2B. The cross-linking fragments were marked and modeled using PyMOL software as shown in Figs. 2C and 2D.

Effect of cross-linking on the advanced structure of Ara h 2

The effect of cross-linking on the secondary structure of Ara h 2 was characterized using far-UV circular dichroism (CD) spectroscopy. As shown in Fig. 3A, the CD spectrum obtained indicated alterations in the secondary structure of cross-linked Ara h 2. The alpha-helical content of Ara h 2 decreased to 16.7%, while that of random coils increased to 34.1%. The β -sheet content of the protein also increased slightly (Table 1). UV absorption spectroscopy could illustrate the environment of aromatic amino acids and help assess the tertiary structure of proteins³³.

The absorption peak of cross-linked Ara h 2 was significantly higher than that of untreated Ara h 2 (Fig. 3B), which indicated that the advanced structure of the protein was modified after PPO-catalyzed cross-linking to expose heterocyclic amino acids.

Ig E binding of cross-linked Ara h 2 products

Peanut-induced anaphylaxis is an Ig E-mediated condition¹. The effect of Ig E binding to PPO-catalyzed cross-linked Ara h 2 was examined via competitive ELISA. As shown in Fig. 4, after cross-linking, the IC₅₀ of Ara h 2 increased slightly. This result indicated that the cross-linking reaction might change the allergenicity of Ara h 2 non-significantly, while the Ig E binding capacity decreased in an indirect ELISA²¹.

***In vivo* allergenicity assessment**

To verify the effect of PPO catalyzed cross-linking on Ara h 2, a mouse model was applied to assess the allergenicity of Ara h 2 before and after the reaction. After exposure to peanut protein, all of the mice groups exhibited significantly higher sIg G and sIg G1 levels compared with that of the PBS control (Fig. 5). Cross-linked Ara h 2 groups showed sIg G levels that were significantly lower than that of the native

Ara h 2 group; sIg G1 levels also changed slightly after the reaction. The presence of sIg G antibodies can be a marker of immune tolerance associated with the activation of regulatory T cells; thus, lower sIg G levels might have an impact on allergic reactions^{34, 35}.

Cytokine levels reflected the intensity of an anaphylactic reaction³⁶. The cytokine levels of the challenged groups were significantly higher (IL-4, IL-13, and IFN- γ) than those of the control groups (Figs. 6A, 6C, 6D), although IL-5 level was not significantly different between groups (Fig. 6B). The IL-13 level of the cross-linked Ara h 2 group was highly significantly lower than that of the native Ara h 2 group; the IFN- γ level of the former was also higher than those of the latter. IL-13 is an anti-inflammatory cytokine that plays a unique role in the induction and maintenance of Ig E production and Ig E-mediated allergic responses^{37, 38}. Reductions in IL-13 indicated that cross-linking might effect the allergenicity of Ara h 2.

Discussion

Ara h 2 could undergo either inter- or intramolecular cross-linking catalyzed by PPO, as speculated by Wu *et al*²¹. PPO-induced cross-linking involves the enzymatic oxidation of phenolic compounds to

o-quinone, followed by cross-linking reactions between tyrosine residues and lysine or arginine residues^{12, 39}. However, lysine residues had not been found in the identified cross-linked peptides of Ara h 2, likely because the protein contains only 3 lysine residues but 21 arginine residues. As such, the likelihood of lysine cross-linking with tyrosine was much lower than that of arginine cross-linking with tyrosine.

The distance between different cross-linking sites and the structure of the cross-linking peptides might exert some impacts on the cross-linking reaction. The intramolecular cross-linking results showed that ARG¹³¹ in peptide c probably cross-linked with TYR⁶⁵ or TYR⁷² in peptide 2. In a molecular model, ARG¹³¹ was at the end of an alpha-helix, while TYR⁶⁵ and TYR⁷² were in random coils. ARG¹³¹ was 23.7 Å from TYR⁶⁵ and 30.1 Å from TYR⁷², which indicated that ARG¹³¹ was more likely to cross-link with the former than with the latter. TYR⁶⁰ in peptide 1 probably cross-linked with ARG¹⁰² or ARG⁹² in peptide b. The spatial distance between TYR⁶⁰ and ARG⁹² (30.4 Å) was similar to that between TYR⁶⁰ and ARG¹⁰² (27.0 Å), but ARG¹⁰² was located on the farther side of an alpha-helix; thus a cross-linking reaction was more likely to process between TYR⁶⁰ and ARG⁹² than between TYR⁶⁰ and ARG¹⁰².

Intermolecular cross-linking might occur between peptides 2 and b and between peptides 3 and a. The ARG¹⁰² residue of peptide b was located on the farther side of an alpha-helix, and other cross-linking sites revealed random coil structures. As all cross-linking sites were located on the surface of the molecule, no reaction site between different residues could be excluded.

The secondary structure of Ara h 2 was markedly altered after cross-linking. Two out of five alpha-helices were included in cross-linking sites, causing a decrease in alpha-helix content and an increase in random coil content. Destruction of the secondary structure of the protein yielded a loose tertiary structure, which exposed heterocyclic amino acid residues and caused a significant increase in the absorption peak of the cross-linked protein compared with that of the native protein⁴⁰. After cross-linking, the spatial structure of Ara h 2 loosened, and the arrangement of surface amino acid residues changed, thereby resulting in a slight decrease in the Ig E binding capacity of the protein²¹.

Ten linear allergen epitopes were indentified in Ara h 2¹⁷, while 7 out of them were involved in cross-linking reactions. This results indicated that cross-linked reaction may directly alter the linear or

conformational epitope, and specifically alter the allergenicity of the protein. The Ig E binding capacity of cross-linked Ara h 2 was assessed by competitive ELISA to determine whether the potential allergenicity of treated Ara h 2 decreased slightly after the reaction¹⁴.

In the mouse model, the sIg G level of the cross-linked protein was significantly lower than that of the native protein. When mice were in a sensitized state, T helper 2 (Th2) cytokine levels were elevated. After exposure to cross-linked peanut proteins, IL-13 levels showed a highly significant decrease while IFN- γ levels increased. IL-13 is produced by Th2-type cells, and the IFN- γ secreted by Th 1 cells could effectively inhibit the Th2 type reaction and reduce Th2 cytokine levels⁴¹⁻⁴³.

Although the linear allergen epitopes were involved in cross-linking sites, the Ig E capacity of the protein did not change significantly. After antigen-presentation, however, the cross-linked protein induced lower sIg G levels and showed reduced sensitization potential. This study demonstrated the potential of precise modification of the structure of proteins by manipulating cross-linking sites to improve their properties.

Overall, there are multiple structural changes which could effect on potential allergenicity. Firstly, the reacting site may alter protein

conformations, which could expose some amino acid residues onto surface, including those in linear epitopes. Secondly, reacting sites closed to linear epitopes may make compact the local structure, which may induce Ig E binding epitope blocking. So after cross-linking reaction, the potential allergenicity of modified protein might not changes directionally .

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Table 1 Contents of the secondary structure of Ara h 2

Samples	α -Helix/%	β -Sheet/%	β -Turn/%	Random coil/%
Native Ara h 2	22.5	25.8	22.4	29.2
Cross-linked Ara h 2	16.7	26.7	22.9	34.1

Figure legends

Figure 1. Tricine–SDS-PAGE of cross-linked Ara h 2 catalyzed by PPO.

M: Markers, lane 1: native Ara h 2, lane 2: PPO-catalyzed cross-linked Ara h 2

Figure 2. Identification of cross-linked sites using mass spectroscopy and StavroX software and the spatial structure of the peanut allergen Ara h 2.02 determined by PyMOL. (A) Information on the identified cross-linked peptides labeled in the mass spectrum. “Cross-linked peptide 1b” means peptide 1 was cross-linked with peptide b; these peptides: 1-3 and a-c were marked in Fig. 2B. 1b and 2c were intramolecular cross-linking peptides, 2b and 3a were intermolecular cross-linking peptides. (B) Amino acid sequence of Ara h 2.02. Here, the boldfaced Y and R in the cross-linkers were possible reaction sites. The cross-linked peptides of R were marked in red, while the cross-linked peptides of Y were marked in blue (neighboring in green). The linear allergen epitopes of Ara h 2.02 were marked with under- and wavy lines. (C) Reaction sites of intramolecular cross-linked Ara h 2. (D) Reaction sites of intermolecular cross-linked Ara h 2. The red and blue areas were cross-linking peptides, and cross-linking reactions occurred between

peptides of the same color. The reaction sites were marked in green or yellow. Distances between intramolecular cross-linked sites were marked by dotted lines.

Figure 3. CD spectrum and UV absorption spectrum of proteins. (A) Effect of cross-linking treatment of Ara h 2 as determined by CD spectroscopy. (B) Effect of cross-linking treatment of Ara h 2 as determined by UV absorption spectroscopy.

Figure 4. Effect of cross-linking on the Ig E binding capacity of Ara h 2 as determined by competitive ELISA. The data indicated mean $\pm$ SD (n = 3).

Figure 5. Relative sIg G and sIg G1 levels detected in sera. The error bar indicates that standard deviation. The data indicated mean $\pm$ SD (n = 3).

*Significant difference ($p < 0.05$) compared with the control, **highly significant difference ($p < 0.01$) compared with the control; ♦significant difference ($p < 0.05$) compared with native Ara h 2.

Figure 6. Cytokine levels in the supernatant of cultured mice spleen cells. (A) IL-4, (B) IL-5, (C) IL-13, and (D) INF- γ . The data indicated mean $\pm$ SD (n = 3). Control = treatment with PBS; error bar = standard deviation.

*Significant difference ($p < 0.05$) compared with the control, **highly

significant difference ($p < 0.01$) compared with the control, $\blacklozenge$ significant difference ($p < 0.05$) compared with native *Ara h 2*; $\blacklozenge\blacklozenge$ highly significant difference ($p < 0.01$) compared with native *Ara h 2*.











