Proteomic and immunological identification of two new allergens from silkworm (*Bombyx mori* L.) pupae

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Abstract

This study explored food allergy caused by eating silkworm (Bombyx mori L.) pupae, a traditionally accepted food and animal feed in East and Southeast Asia, and identified two new allergens by proteomic and immunological methods. Proteins isolated from silkworm pupae were separated by two-dimensional gel electrophoresis (2-DE); pooled sera from patients allergic to silkworm pupa proteins were used to detect immunoglobulin E (IgE)-binding proteins by western blotting, and allergens specific for silkworm pupa consumption-caused allergy were visualised with the ECL reagents. The selected allergen proteins were further identified by matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) analysis. Finally, chitinase and paramyosin were identified as silkworm pupa proteins showing strong immunoglobulin (IgE)-binding reaction. Analysis of the sequence homology of the two proteins using the AllergenOnline database indicated that chitinase and paramyosin shared 24.8% and 62.8% sequence homology with known allergens Der f 18 (Dermatophagoides farinae) and Der p 11 (Dermatophagoides pteronyssinus), respectively. Our results shed light on the understanding and treatment of silkworm pupa allergy.

Key words: silkworm (Bombyx mori L.) pupa, allergen, chitinase, paramyosin, western blot, MALDI-TOF-MS.

Introduction

Food allergies characterised by an acute onset of immunoglobulin (Ig) E-mediated immune-response symptoms are a growing public health concern in the world [1]. Approximately 12 million Americans suffer from food allergy, and a similar prevalence of food allergy appears in European countries [2]. Commonly, food allergy symptoms can occur within minutes to hours of ingesting the triggering food and can vary in severity from mild rashes, itching, swelling, etc. to life-threatening anaphylactic shock (AS), lethal anaphylaxis, etc. [3]. It has been reported that more than 170 different foods in the world could cause allergic reactions; among them, eight foods, including milk, eggs, fish, shellfish, wheat, tree nuts, peanuts, and soybeans, cause the majority (approximately 90%) of reported allergic reactions [4]. Common food allergens vary between geographic regions and food source. In European countries, significant allergens may be celery, mustard, sesame, lupine, and molluscan shellfish, while in Japan buckwheat is a common major allergen [3].

Sericulture has been a well-established rural agribusiness for a quite long time in more than 70 countries and regions in the world, and it still plays an important role in rural areas in China and India, among many other developing countries [5]. Silk production is the primary goal of sericulture, contributing more than 42 billion US dollars in economic value globally in 2010, of which nearly 74% was produced in China [6]. The silkworm pupa is one of the main by-products in sericulture, and it has been used as food, medicine, and animal feed in China for quite a long time. Owing to the popularity of silkworm pupa- and moth-eating habits, a distinctive diet culture even formed in most regions of China. Therefore, food safety issues caused by silkworm pupa consumption have drawn public attention recently. Although there have been many reports on silkworm allergies [7], little information is available on the specific allergens from silkworm pupae. The aim of the present study was to identify specific protein components of silkworm pupa protein that are potentially responsible for the allergenic response in humans.

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Material and methods

Materials

Silkworm (*Bombyx mori* L.) pupae were obtained from silkworm germplasm bank of the Sericultural and Agro-Food Research Institute, Guangdong Academy of Agricultural Sciences. The silkworm pupae were snap frozen in liquid nitrogen and stored at –78°C until used. Biotinylated goat anti-human IgE antibody and peroxidase-labelled streptavidin were purchased from KPL (Gaithersburg, MD, USA). The Bradford assay kit and PVDF immunoblot membrane were from BioRad (Hercules, CA, USA). The skimmed milk powder was purchased from the local supermarket. All materials were obtained from Sigma (USA) unless stated otherwise.

Human sera

Sera from four patients were obtained from Daliushu Branch, Weifang the Fourth Hospital. The patients were all selected with a documented clinical history of immediate hypersensitivity reactions to silkworm allergen. All sera were obtained from patients, who suffered a serious allergy after ingestion of silkworms. These patient sera were used as a pooled positive sample. All manipulations were approved by each patient. All sera were pooled and divided into portions and stored at –78°C until used.

Preparation of soluble antigens from silkworm pupa

The extraction of soluble allergens from silkworm pupae was performed in our lab. Briefly, fresh silkworm pupae were washed thoroughly to remove residual impurity. Silkworms were cut into small chunks with a meat grinder (Zhuhai Yingbiao Machine Co., Guangdong, China) and then placed in a pre-cooled mortar with liquid nitrogen. Liquid nitrogen was added rapidly to the mortar until the pupa chunks were ground into powder without significant particles. The powder was then dissolved in 800 μl ALK lysis buffer (containing 7 M urea, 2 M thiourea, 2% CHAPS, and 20 mM tris) and sonicated. The lysates were cooled on ice and then centrifuged at 12,000 rpm for 20 minutes at 4°C. Each Eppendorf tube (1.5 ml volume) was added with 250 μl of the supernatant and then 1 ml of acetone to precipitate proteins at –20°C overnight. Finally, the tubes were centrifuged at 12,000 rpm for 20 minutes at 4°C to collect the pellets (soluble antigens), which were placed on a clean towel for natural drying and then stored at –80°C before further use. The total protein of the sample was quantitated using the Bradford assay kit [8].

Procedure of 2-DE and western blot

Silkworm extracts (100 μg) were separated by 2-DE, as described previously [9]. Briefly, the protein sample was solubilised in rehydration buffer containing 7 M urea (Amersham), 2 M thiourea (Amersham), and 4% CHAPS. The solution was loaded onto an Immobiline 17-cm IPG strip with pH 4-7 (Bio-Rad, USA) and focused to the isoelectric points by an Ettan IPGphor 3 apparatus (GE Healthcare, Sweden). The strip was equilibrated in SDS equilibration buffer (with 50 mM tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, and 0.002% bromophenol blue) before separating the focused proteins in the second dimension by SDS-PAGE. This experiment was performed in duplicate for each sample, and one set of gels was dyed with Coomassie brilliant blue R-250 solution (50% ethanol, 10% acetic acid, and 40% water) overnight, whereas the other gel was processed for further western blot analysis. For western blot, the gel was transferred to nitrocellulose membranes in TBS buffer (pH 8.8). After blocking in TBS buffer with 1% Tween-20 (TBST) containing 5% skimmed milk, the membrane was incubated with pooled sera diluted 1 : 250 (v/v) on a rocker at 4°C overnight, then washed three times with TBST buffer, and incubated with biotinylated goat anti-human IgE antibody and peroxidase-labelled streptavidin for two hours. The membrane was then washed three times with TBST, each for five minutes, and detected using the ECL Western Blot kit (Beijing CoWin Biotech, Beijing, China) according to the manufacturer's instructions. Based on the results of immunoblot, the positive spots on 2-DE gel were excised, and the proteins were subjected to MS analysis.

MALDI-TOF-MS analysis

Protein spots corresponding to allergens identified on western blot were excised manually from 2-DE gels. The enzymolysis procedure of target spots was referred to the previous description [9]. The vacuum-dried peptide mixtures enzymolysed from selected protein spots were dissolved in 1.5 μl resolve solution containing Milli-Q water, 30% acetonitrile (ACN), and 0.1% trifluoroacetic acid (TFA). Then, 0.8 μl of the mixture was sucked and dropped onto an alpha-cyano-4-hydroxycinnamic acid (HCCA) matrix substance, dried at room temperature in a fume cupboard, and analysed by MALDI-TOF-MS on a mass spectrometer (Bruker Dalton, Germany). The UV laser was operated at a 200 Hz repetition rate with a wavelength of 355 nm. The acceleration voltage was operated at 20 kV, and the mass resolution was maximised at 1,500 Da. BioTools (Bruker Dalton, Germany) software was used to filter the signal baseline peak, distinguish the signal peak, and search for the peptide and protein on the National Centre for Biotechnology Information (NCBI) databases. Protein identification was carried out by peptide mass fingerprint (PMF) using Mascot software. Protein scores greater than 52 were assumed to be significant ($p < 0.05$).

Results

Comparative proteomic analysis by 2-DE and western blot

Water-soluble proteins from the dazao strain of silkworm pupa were separated by means of 2-DE with immobilised pH gradients (IPGs) and SDS-PAGE. Figure 1A shows the spots of water-soluble silkworm pupa proteins in a typical 2-DE map. More than 150 different protein spots were fractionated in the 2-DE experiment in the range 10-170 kDa and pH 4-7 with silver staining. Most proteins in the map had a pI under pH 6.0 and a molecular mass of 10-130 kDa. As shown in Figure 1B, the same 2-DE strip of water-soluble silkworm pupa proteins stained with Coomassie brilliant blue was compared with western blot to select spots for allergen identification. There were 11 spots showing specific IgE reactivity with pooled sera from the patients; of them, 7 spots (1-7) had remarkable immunoblotting signals,

while the other 4 (8-11) displayed relatively weak reactivities. Therefore, spots 1-7 were selected for further characterisation by peptide sequencing. The serum of a non-allergic patient did not show any IgE reactivity (see Fig. 1C).

MALDI-TOF-MS analysis

The selected protein spots were excised from the gel and analysed by MALDI-TOF-MS. By comparison with the established peptide mass fingerprint database, a protein score > 50 with several matched peptides could be considered as a significant identification [10]. In the peptide mass fingerprint analysis, the masses obtained from the excised protein spots 1-4 showed the highest correlation with chitinase from *Bombyx mori* L., with a peptide sequence coverage of 14-25% and sequence scores of 61-197. Meanwhile, protein spots 5-7 showed the highest correlation with paramyosin from *Bombyx mori* L., with a peptide sequence coverage of 28-35% and sequence scores of 225-341 (Table 1).
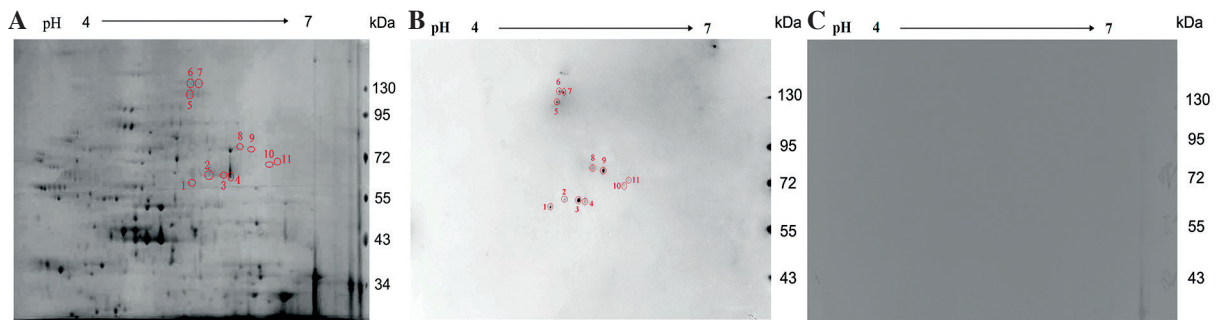
Fig. 1. Identification of IgE-binding antigens extracted from silkworm (*Bombyx mori*) pupae. **A)** 2-DE stained for total protein analysis. **B)** Western blot analysis of IgE reactivity to silkworm soluble antigens using pooled serum. **C)** Western blot analysis of IgE reactivity to silkworm soluble antigens using normal serum. The red circle in the figure shows the IgE-binding protein that was sent for MALDI-TOF/MS analysis. Molecular weight (MW) in daltons is indicated in the right margin of the gel

Table 1. MALDI-TOF/MS identification of selected protein spots from peptide mass fingerprint database

Spot No.	Protein description	Top score	Sequence coverage	Mass and pI	Number of matched regions
1	chitinase	197 for gil169234932	25%	61519/5.78	55-75, 154-167, 212-220, 236-245, 286-293, 339-350, 411-441, 465-475, 493-514
2	chitinase	61 for gil169234932	16%	61519/5.78	55-75, 336-350, 411-420, 431-441, 465-475, 493-514
3	chitinase	196 for gil169234932	14%	61519/5.78	154-167, 339-350, 411-420, 431-441, 465-475, 493-514
4	paramyosin	120 for gil195963325	15%	102803/5.43	177-193, 252-269, 383-397, 486-494, 572-588, 589-602, 627-638, 690-701, 852-871
5	paramyosin	255 for gil195963325	28%	102803/5.43	42-51, 60-90, 231-242, 252-269, 289-307, 383-397, 439-446, 448-456, 486-494, 513-525, 571-602, 604-623, 627-638, 690-701, 836-847, 852-871
6	paramyosin	341 for gil195963325	35%	102803/5.43	42-51, 60-74, 62-90, 231-242, 252-279, 289-316, 370-376, 383-397, 409-417, 439-456, 486-394, 505-525, 571-602, 604-623, 627-638, 690-701, 807-821, 836-847, 852-871
7	paramyosin	225 for gil195963325	30%	102803/5.43	42-51, 60-90, 231-241, 252-279, 289-307, 383-397, 448-456, 486-494, 513-525, 572-602, 604-623, 627-638, 690-701, 807-821, 836-847, 852-871

Fig. 2. Multiple alignment of amino acid sequences by T-Coffee (www.tcoffee.org) between new silkworm allergens and known allergens of similar type. A, silkworm chitinase with Der f 18 (*Dermatophagoides farinae*) (UniProt entry: Q86R84); B, silkworm paramyosin with Der p 11 (*Dermatophagoides pteronyssinus*) (UniProt entry: Q6Y2F9). Colour shades indicate levels of amino acid homology between aligned sequences

Therefore, the protein spots 1-4 and spots 5-7 represented, respectively, two different proteins. According to former reference on peptide mass fingerprint analysis [11, 12], the same kind of protein on the 2-DE gels can be characterised by multiple points, on behalf of the modification, subunits, or peptide fragments of protein. Therefore, we speculated that these two clusters of protein spots may have a certain extent of modification in the protein structure. Hence, these two proteins, chitinase precursor and paramyosin, with the highest Mascot scores were identified as allergens of silkworm pupa.

Sequence analysis for potential allergens

Recent studies showed that chitinases and chitinase-like proteins are only present in lower life forms, but they are also noticed in higher life forms, e.g. mouse and human [13]. In addition, chitinases and chitinase-like proteins were also shown to play an important role in allergy [13, 14]. However, there is little information on chitinase allergen from *Bombyx mori* L., which thus warrants more efforts in further investigation. Paramyosin is a myosin filament-related protein found in striated muscle of invertebrates, which plays an important role in the process of myosin filament assembly [15] and mainly acts as a major muscle component in invertebrates. In addition, paramyosin was reported to possibly regulate the host immune responses and further induce allergenic reactions in humans [16], suggesting that paramyosin is a kind of potential protein allergen. In order to validate the extent of homologies between the silkworm allergens and their homologous proteins identified as allergen from other sources, we compared the amino acid sequences of these two proteins against existing allergens in the AllergenOnline (http://www.allergenonline.org) database of the Food Allergy Research and Resource Program (FARRP) [17]. As a result, we found that silkworm chitinase closely resembles Der f 18 of *Dermatophagoides farinae* (Q86R84) (24.8% amino

acid identical and 57.4% similar), and that silkworm paramyosin closely resembled Der p 11 of *Dermatophagoides pteronyssinus* (Q6Y2F9) (62.8% amino acid identical and 90.0% similar). Figure 2 shows the multiple alignment of amino acid sequences of silkworm chitinase and paramyosin with those of the allergenic chitin-binding protein Der f 18 (Fig. 2A) and Der p 11 (Fig. 2B), respectively, by T-Coffee (www.tcoffee.org) method, which indicated that the two silkworm allergens shared identical and similar amino acid sequences with their corresponding homologous allergenic proteins.

Silkworm pupae have been regularly consumed in China, India, and some other traditional sericultural countries for a long time. Health problems may thus emerge due to the utilisation of silkworm pupa protein. However, to date, only a few proteins have been characterized as major allergen from silkworm larvae [18, 19]. Here we identified two putative allergenic proteins chitinase and paramyosin by specific IgEs from the sera of patients allergic to silkworm pupa protein. Through multiple sequence alignment of amino acid sequences of silkworm allergen with the allergen database, we speculated that silkworm chitinase and paramyosin might be cross-reactive allergens of house dust mites (Der f 18 from *Dermatophagoides farinae* and Der p 11 from *Dermatophagoides pteronyssinus*). Further studies are needed to identify the specific epitopes of these two potentially allergenic proteins.

Conclusions

The present study identified and partially characterised two novel allergens of *Bombyx mori* L., the chitinase and paramyosin, by 2-DE, Western blot, and MALDI-TOF-MS approach. Further research is required to determine the specific epitope and biological function of these two proteins. In addition, the potential cross-reactivity between silkworm chitinase and paramyosin and the corresponding homologues Der f 18 and Der p 11 also warrants further exploration. To our knowledge, this is the first study focusing on identification of allergens from silkworm pupa protein using sera from patients seriously allergic to cooked silkworm pupae. Further investigations may provide valuable insight into the silkworm pupa allergy.

The authors declare no conflicts of interest.

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