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Identification of a serine protease inhibitor homologue in Bird's Nest by an integrated proteomics approach

For centuries, the edible nests of *Collocalia* spp. ("Bird's Nests") have been used as a Chinese delicacy that had been claimed to be an effective health-giving tonic. However, clinical studies indicated that in Singapore, Bird's Nest is the most common cause of food-induced anaphylaxis in children, which could lead to potentially life-threatening allergenic reactions. The purpose of this study was to characterize the major allergens in Bird's Nest by using the combined technologies of two-dimensional gel electrophoresis (2-DE), immunochemistry, *N*-terminal protein sequencing, and mass spectrometry. Results from the immunostaining of the Western blots of the Bird's Nest 2-DE separated proteins with the sera from allergic patients indicated the presence of a major allergen of 66 kDa. Initial searches of the matrix assisted laser desorption/ionization – time of flight – mass spectrometry (MALDI-TOF-MS) tryptic peptide masses of the allergen in the SWISS-PROT and NCBI nonredundant databases revealed that this protein was novel. Based on the partial protein sequence information obtained from *N*-terminal microsequencing and nanoelectrospray-tandem MS, the 66 kDa immunoreactive allergen was found to be homologous to ovomucoid, a Kazal-type serine protease inhibitor, which is one of the dominant allergens found in chicken egg white.

Keywords: Bird's Nest / Allergen / Immunoreaction / Two-dimensional gel electrophoresis / Edman degradation / Mass spectrometry

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1 Introduction

The edible nests of swiftlets (*Collocalia* spp.) are made of the male birds' saliva during the breeding season [1, 2]. For centuries, these nests have been used as a Chinese delicacy known as Bird's Nest (BN) that had been claimed to have medicinal value, and each year a large quantity of BN is consumed mainly by the ethnic Chinese. However, clinical studies in Singapore indicated that BN was the most common cause of food-induced anaphylaxis in children, which could be life-threatening [3, 4]. The symptoms were typical of a Type I hypersensitivity (angioedema, wheezing, urticaria, and abdominal cramps). Clinically, the most effective means of managing food-induced anaphylaxis is to recognize the incriminating food allergen. But to date, little study has been done on the natural history and the characterization of BN allergens.

Two-dimensional gel electrophoresis (2-DE) and immunoglobulin E (IgE)-immunoautoradiography have been applied for the characterization of major chicken egg white allergens, but the approach was limited to the identification of known proteins [5]. With the advent of proteomics, the development of protein database search and identification algorithms for mass spectrometry data [6, 7], the process of characterizing proteins separated by 2-DE has been greatly simplified and accelerated. Here, we report the isolation and identification of a novel BN allergen protein by using the combined technologies of 2-DE, immunochemistry, *N*-terminal protein sequencing and mass spectrometry. The results suggested that the BN allergen was homologous to ovomucoid, a Kazal-type serine protease inhibitor in egg white.

2 Materials and methods

2.1 BN extract preparation

The commercially available edible nests of *Collocalia* spp. ("Bird's Nests") were from Sarawak, Malaysia. Crude extracts of the nests were made by aqueous extraction. Feathers in the nests were first manually removed. The nests were then pounded in a mortar, suspended in distilled deionized water, and allowed to elute for 24 h at 4°C.

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Abbreviations: BN, Bird's Nest; IgE, immunoglobulin E; nESI, nanoelectrospray ionization

The suspension was centrifuged and the supernatants subsequently lyophilized. These were stored at -80°C prior to protein analysis.

2.2 2-DE

The BN extract was thawed to room temperature and dissolved in a cocktail of 9 M urea (Bio-Rad Laboratories, Hercules, CA, USA), 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS; USB, Amersham Pharmacia Biotech AB, Uppsala, Sweden), 40 mM tris(hydroxymethyl)aminomethane (Tris; J. T. Baker, Phillipsburg, NJ, USA), and 1 mM phenylmethylsulfonyl fluoride (PMSF; Sigma, St. Louis, MO, USA). 2-DE was performed essentially according to Seow *et al.* [8]. The amount of protein loaded was approximately 400 μg protein per gel. The first dimensional IEF was performed on 13 cm pH 4–7 linear IPG strips (Amersham Pharmacia) at 20°C with a maximum current setting of 50 μA /strip using an IPGphor IEF unit (Amersham Pharmacia). SDS-PAGE was performed on 1.0 mm thick 13% polyacrylamide gels at a constant current of 30 mA/gel at 10°C using a Bio-Rad Protean II xi electrophoresis unit.

2.3 Western blotting

The preparation of BN-specific IgE was conducted as previously described [9]. The 2-DE separated BN proteins were transferred for 2 h at 150 mA onto a nitrocellulose (NC; Hybond-C extra; Amersham Pharmacia) membrane. These were blocked with 3% nonfat milk in 0.05% PBS-Tween (PBST) for 1 h and then incubated with sera from patients sensitive to BN-induced anaphylaxis and normal sera as control for 18 h at 4°C . After washes with PBST, the strips were incubated for 1 h at room temperature with a mouse-biotinylated anti-human IgE antibody (2 $\mu\text{g}/\text{mL}$). The strips were washed with PBST again and then incubated for 1 h at room temperature with Extravidin peroxidase conjugate (Sigma; 1:2000 PBST). The strips were washed with PBST and developed using ECL-plus (enhanced chemiluminescence) according to manufacturer's recommendations. For protein *N*-terminal sequencing, the 2-DE separated BN proteins were electroblotted onto polyvinylidene difluoride (PVDF) membranes and visualized with Coomassie Brilliant Blue G-250 [6, 10]. For mass spectrometry analyses, the 2-DE separated BN proteins were silver-stained. Gel spots corresponding to the IgE detected allergens were excised manually with a scalpel, digested with trypsin, and subsequently subjected to mass spectrometric analyses.

2.4 *N*-terminal microsequencing

Each individual Coomassie Brilliant Blue G-250 stained PVDF protein spot was placed directly onto a Procise 494 cLC capillary protein sequencer (Applied Biosystems, Foster City, CA, USA) for automatic Edman degradation analyses according to the manufacturer's standard protocol.

2.5 MS analysis

Peptide mass fingerprint mapping was performed using a PerSeptive Biosystems Voyager-DE STR MALDI-TOF mass spectrometer (Framingham, MA, USA) according to a previously published method [8]. *De novo* peptide sequencing was performed using an AB/MDS Sciex QSTAR tandem hybrid QqTOF MS system (Concord, ON, Canada) equipped with an nESI source (Protana, Odense, Denmark). The tryptic digested protein samples were individually desalted and concentrated with C_{18} Zip-Tips (Millipore, Bedford, MA, USA) and finally eluted with 2 μL of 60% methanol/0.1% formic acid. Sample (2 μL) was loaded onto a spray needle and the spray was initiated by applying a spray potential of 850 V. The QSTAR system was calibrated once a day and operated with resolution of about 10 000 full width at half maximum (FWHM). Data acquisition was carried out using the "TOF-Tune" software and data processed using the "Biomulti-view" software (AB/MDS Sciex). The *y*" and *b*" ions weightage was used to obtain sequences from the MS/MS analysis of the peptides. In these sequences, cysteine was modified as a carboamidomethyl derivative while methionine was in the oxidized form. Since glutamine and lysine had equivalent molecular masses, they were not differentiated.

2.6 Protein database search

Protein identification by peptide mass mapping was conducted by searching the SWISS-PROT and NCBI nonredundant databases using the MS-Fit program (Protein Prospector; UCSF, San Francisco, CA, USA) with the previously described parameters [8]. Partial sequence data obtained from *N*-terminal protein sequencing and MS/MS peptide sequencing were used to search for homologous proteins with the BLAST program from NCBI. Parameters used for BLAST searches were: blastp program, nr database, PAM30 matrix, and default values for the remaining settings.

3 Results and discussion

3.1 Immunoblotting and *N*-terminal sequencing

The 2-DE pattern of BN extract was relatively simple, with only a few major proteins (Fig. 1). After the immunoblotting step, a chain of seven spots was seen in the 66 kDa region of the NC membrane (Fig. 2a) as compared to the negative control (Fig. 2b). In a parallel experiment in which electroblotting was carried out using PVDF membrane, corresponding protein spots were excised for *N*-terminal amino acid sequencing. The major protein spot (#5) had an *N*-terminal amino acid sequence of VEVDCS-QYSSDVTKDGTVRV (peptide 1 in Table 1). All the remaining spots had the same *N*-terminal sequences of VEVDCS, suggesting that they might be isoforms of the protein. The result of a BLAST protein database search

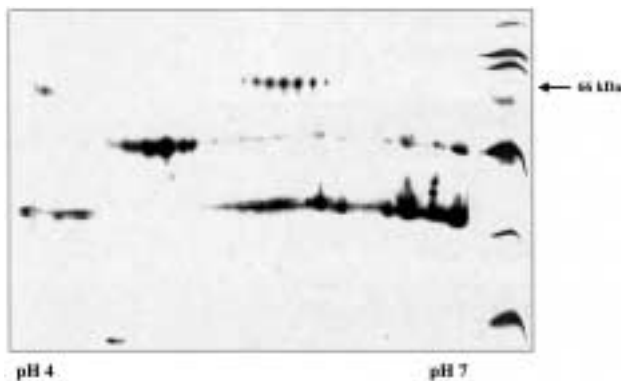


Figure 1. Preparative 2-D map of the BN extract. Approximately 400 μ g of protein lysate was focused on a pH 4–7 linear IPG strip before separation on a 10% polyacrylamide gel. Silver staining was used for detection of protein spots. A chain of seven 66 kDa protein spots were visualized.

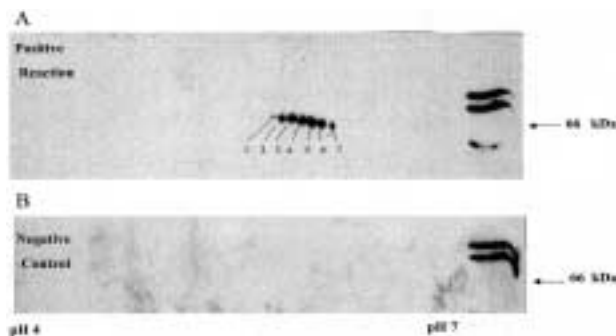


Figure 2. 2-DE immunoblotting with extracted BN proteins. The NC membrane was probed with (A) sera from patients sensitive to BN allergen and (B) normal sera as negative control. A chain of seven protein spots (66 kDa) was visualized when the NC reacted with sera of patients sensitive to BN allergens.

indicated that the allergen protein was highly homologous to the ovoinhibitor precursor found in chicken (IOV7_CHICK, P10184, Table 1A).

3.2 MS

To make a more affirmative confirmation of the identity of the 66 kDa allergen, mass spectrometric analyses were carried out on the in-gel digested tryptic peptides of protein spot #5. The peptide mass fingerprint results derived from MALDI-TOF-MS analysis was used to search against the SWISS-PROT and NCBI nonredundant databases, but no protein match was returned (data not shown). Thus, *de novo* peptide sequencing was carried out using nESI-MS/MS to obtain peptide sequences for database search. The nESI-TOF-MS spectrum for the tryptic peptide mixture of the 66 kDa allergen is shown in Fig. 3, and six doubly charged peptides with m/z 829.8, 808.8, 733.9, 728.8,

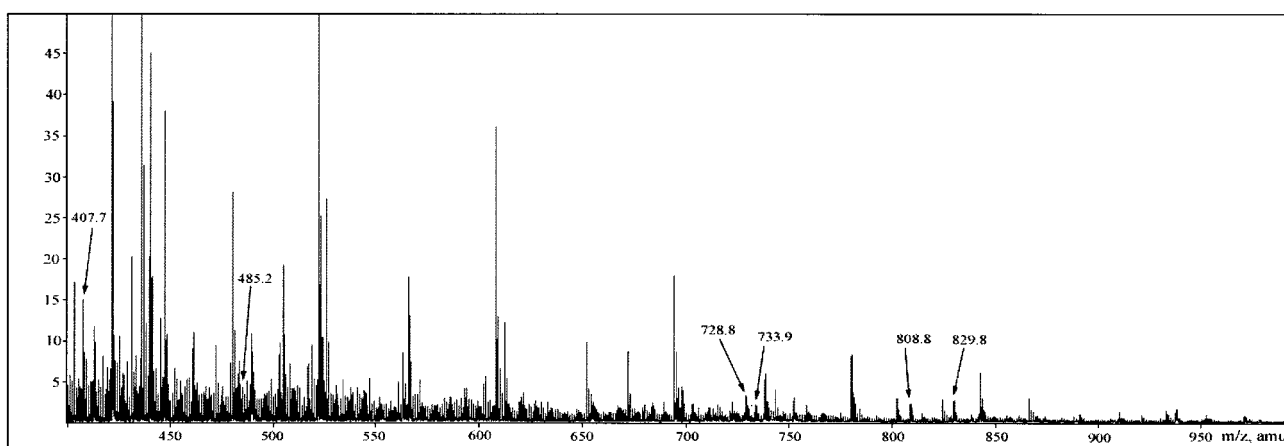


Figure 3. nESI-TOF-MS spectrum of the BN allergen protein. The data were processed using the “Biomultiview” software (AB/MDS Sciex). MS spectrum of peptide mixture was obtained from a tryptic in-gel digestion of the 2-DE separated 66 kDa protein. Six doubly charged peptides were used for fragmentation in the collision cell to obtain sequence information.

485.2, and 407.7, respectively, were selected for peptide sequencing. The MS/MS spectra for two of these peptides (m/z 808.8 and 829.8) are shown in Fig. 4 and 5. In all the MS/MS data, cysteine was identified as a carboamidomethyl derivative since iodoacetamide was used for protein alkylation. In both of these peptides, residue Q was called in favor over K since it was not cleaved by trypsin. The complete amino acid sequences of four of the peptides and the *N*-terminal amino acid sequence of the allergen (as determined by Edman degradation) and their alignments with chicken ovomibitor precursor are summarized in Table 1A. The other two doubly charged peptides (m/z 407.7 and 485.2) had the sequences of VLVACPR and HVLVCPR, respectively, and it is apparent that these are fragments of peptide 2 (Table 1A).

The sequence agreement between the 66 kDa allergen peptides and the corresponding chicken ovomibitor peptide region (spread over five domains), is obvious since the homology and identity values were in the region of 75–90% and 54–80%, respectively. In addition, the

cysteiny residues in the BN allergen also matched the conserved cysteiny residues in the five domains of ovomibitor. The relative positions of these peptides in the complete amino acid sequence of chicken ovomibitor precursor are shown in Table 1B. Chicken ovomibitor (excluding its signal sequence peptide 1–23) contains 448 amino acid residues and should have a calculated molecular mass of 49, 405 Da, and a theoretical isoelectric point (pI) of 6.07. It has been shown that the BN proteins are heavily glycosylated [12], and we have found that the BN allergen reacts with five of the nine anti-lectins (anti-*Arachis hypogaea* lectin, anti-*Canavalia entiformes* lectin, anti-*Riunns communis* lectin, anti-*Phaseolus vulgaris* lectin and anti-*Glycine max* lectin) (data not shown), hence the discrepancy in the molecular mass between them could be due to the presence of the glycan chains. Nevertheless, the experimental pI of the BN allergen is in agreement with the calculated pI of the chicken ovomibitor. These results suggest strongly that the 66 kDa BN allergen reported here is a homologue of chicken ovomibitor, which belongs to the Kazal inhibitor family.

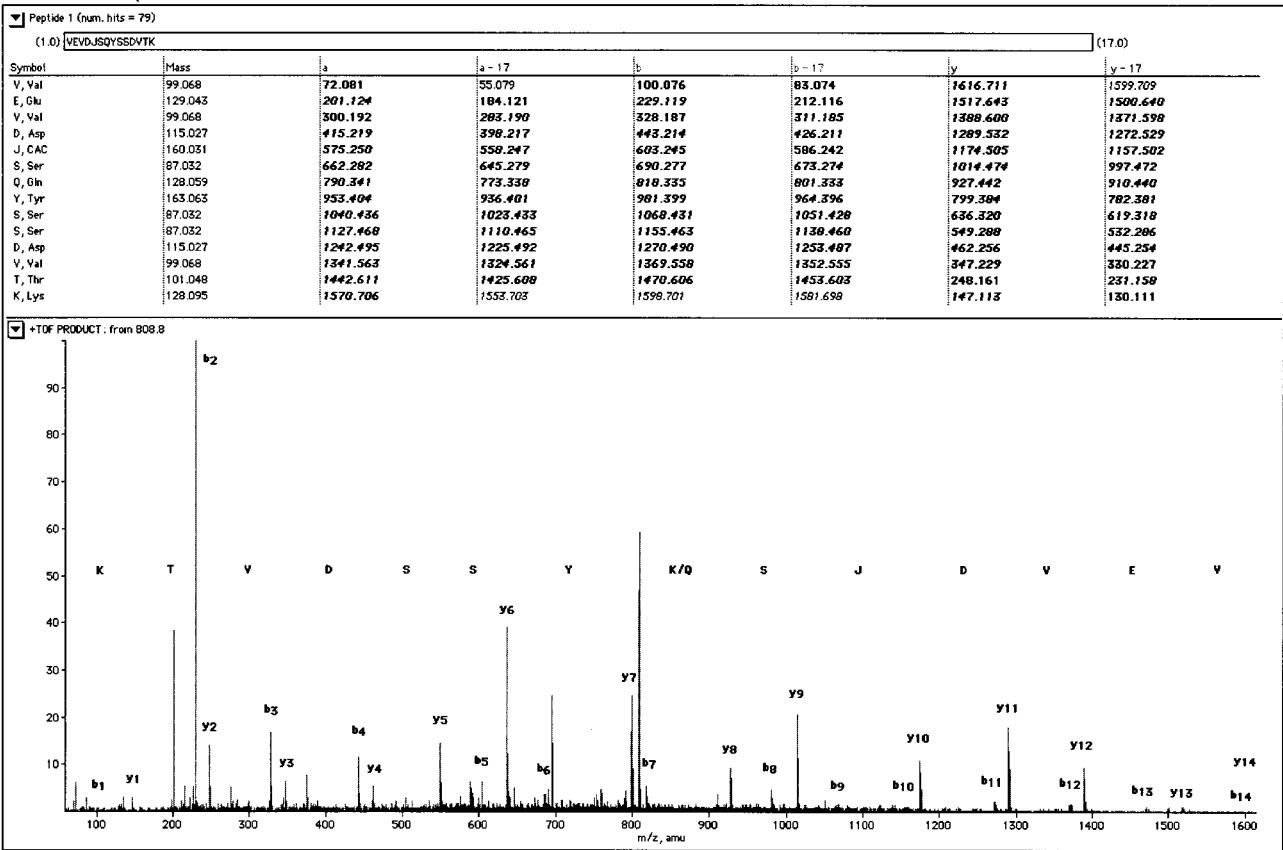


Figure 4. nESI-TOF-MS/MS spectrum of the BN allergen protein. The data were processed using the “Biomultiview” software. MS/MS spectrum of the doubly charged ion (m/z 808.8) shows complete b⁺- and y⁺-series corresponding to the sequence VEVDSQYSSDVTK. J represents the carboamidomethyl derivative of cysteine. Q and K have equivalent molecular masses but the amino acid in this peptide favors Q. Database searches revealed the peptide to be homologous to ovomibitor precursor in chicken (SWISSPROT: IOV7_CHICK, P10184).

Table 1B. Coverage of amino acid sequences obtained from BN allergen (highlighted and underlined) with the chicken ovoinhibitor precursor

(I)		(II)	
1	mrtarqfvqv alalccfadi afg <u>IEVNCSL</u> <u>YASGIGKDG</u> T <u>SWV</u> ACPRNL PVC GTD GSTY SNECGICLYN REHGANVEKE YDGECPKHV MIDCSPYLQV	100	
(III)		(IV)	
101	<u>VRD</u> GNTMVAC <u>PR</u> ILKPVC GS DSFTYDNECG ICAYNAEHT NISKLHDGEC KLEIGSVDCS KYPSTVSK <u>DG</u> <u>RTL</u> VACPRIL SPVCGTDGFT YDNECGICAH	200	
(V)		(VI)	
201	NAEQRTHVSK KHDGKCRQE <u>I</u> <u>PEIDCDQYPT</u> RKTGGKLLV RCPRILLPVC GTD GFTYDNE CGICAHNAQH GTEVKKSHDG RCKER <u>STPLD</u> <u>CTQYLS</u> NTQN	300	
(VII)		(VIII)	
301	GEAITACPI LQEVCGTDGV TYSNDCSLCA HNIELGTSVA KKH DGRCEE VP ELDCSKYK TSTLKDGRQV VACTMIYDPV CATNGVTYAS ECTLCAHNLE	400	
401	QRTNLGKRKN GRCEEDITKE HCREFKQVST ICTMEYVPHC GSDGVYSNR CFFCNAYVQS NRTLNLVSMA AC	472	

Positions 1–23 are signal sequences. Seven domains of chicken ovoinhibitor are indicated in Roman numerals.

There is a wealth of literature on the allergenicity of the two major egg white proteins, ovomucoid and ovoinhibitor, in chicken [14–16]. These proteins consist of three and seven domains, respectively, and are topologically homologous [17]. It has been reported that ovoinhibitor possesses antigenic determinants that are conformationally homologous with those of ovomucoid and cross-reacts with anti-ovomucoid antibodies [18]. Clinical reports have shown that egg white proteins, especially ovomucoid, have an important role in the pathogenesis of allergic reactions [19, 20]. They may act not only as ingested allergens but also as aeroallergens [21]. Our finding that the 66 kDa allergenic protein from BN is a homologue to a member of this class of allergenic proteins seems to concur with these clinical findings.

4 Concluding remarks

The combination of immunology and proteomics technology has opened a new avenue for the isolation and characterization of novel allergen proteins. After the detection of the allergens via IgE reaction, corresponding protein spots could be subjected to protein analysis. Peptide mass fingerprinting was not suitable for novel protein identification because peptide masses were affected by amino acid variations, while the partial peptide sequence information from N-terminal protein sequencing and MS/MS could be used to search databases and look for homology. The results of this study have given us more insight into the allergenic properties of BN proteins and may lead to the development of reagents for prevention and treatment of BN allergy. Recently, there was an interesting report claiming that organic arsenic intoxication might be the cause of anaphylaxis after consumption of BN [22]. Thus, further characterization of this protein would be important.

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