

Immunochemical characterization of edible bird's nest allergens

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Background: We have previously described anaphylaxis induced by edible bird's nest (BN) and demonstrated that this condition is IgE mediated.

Objectives: This study aimed at describing the immunochemical properties of the BN allergens. Comparative studies between 3 commercially available sources (according to the country of origin) of BN were also made.

Methods: Crude extracts of commercially available processed BN from Sarawak (Malaysia), Thailand, and Indonesia and fresh unprocessed BN from the caves of Sarawak were obtained by means of aqueous extraction. Specific IgE toward these sources were determined by using fluorescence allergosorbent tests (FASTs). Cross-reactivity studies between the 3 sources of commercially available processed BN were carried out by means of FAST inhibition. Immunochemical characterization by means of IgE immunoblot, periodate treatment, and heat stability studies were carried out on fresh unprocessed BN from Sarawak.

Results: Serum from allergic patients showed differences in IgE binding to the 3 sources of commercially available BN, with the highest levels of specific IgE recorded with the Sarawak source ($P < .0001$). Of these, only the Sarawak and Thailand sources showed considerable cross-reactivity. Further work on the unprocessed fresh Sarawak source identified a putative 66-kd major allergen containing several isoforms. Periodate treatment resulted in loss of IgE binding. Despite a progressive decline in the molecular weights of allergens on SDS-PAGE with increasing periods of boiling, IgE binding, as assessed by means of FAST, was not affected. N-terminal sequence of the major putative allergen (66 kd) showed homology to a domain of an ovomucoid precursor in chicken (SWISS-PROT accession No. P10184).

Conclusions: We have described the immunochemical properties of BN allergens. Edible BN from different sources are allergenically dissimilar. The putative major allergen is a 66-kd protein. (J Allergy Clin Immunol 2001;107:1082-8.)

Key words: Collocalia species, swiftlets, bird's nest, anaphylaxis, allergen, IgE, protein, food allergy, immunochemical

Abbreviations used

BN: Bird's nest

FAST: Fluorescence allergosorbent test

PBST: 0.05% PBS-Tween

The nests of 4 species of swiftlets (*Collocalia* species) have been harvested for human consumption for centuries. These 4 species are *Collocalia fuciphaga*, *Collocalia germanis*, *Collocalia maxima*, and *Collocalia unicornis*.¹ These birds are found only in the southeast Asian region. The nests built by male swiftlets during breeding season are made almost entirely from saliva produced by the bird's sublingual salivary glands. Some species also include feathers in their nests, but this amounts at most to 10% of the dry weight. The nests are composed mainly of glycoproteins. The carbohydrate component consists of 9% sialic acid, 7.2% galactosamine, 5.3% glucosamine, 16.9% galactose, and 0.7% fucose. The most abundant amino acids present are serine, threonine, aspartic acid, glutamic acid, proline, and valine.² One study has shown the presence of a glycoprotein capable of promoting cell division, and another has demonstrated the presence of an epidermal growth factor-like protein.^{3,4}

These nests are usually double boiled with sugar to make a delicacy known as bird's nest (BN; known in Chinese as yen wo) and is a highly esteemed food tonic believed to have medicinal value. World trade figures estimate that in 1989, 162,896 kg of nests were exported, amounting to a value of \$130,000,000 Hong Kong dollars. Hong Kong is the world's largest consumer, with the ethnic Chinese of North America being the second largest market. Demand is so high that the birds are in danger of extinction.¹

BN allergy was the most common cause of anaphylaxis reported to our hospital (National University Hospital), surpassing other well-defined food allergens, such as cow's milk or egg in younger children and peanut or crustacean seafood in older children.⁵ We have previously described the clinical aspects of BN-induced anaphylaxis.⁶ The objective of this project was to evaluate the immunochemical properties of these allergens, including the allergenicity of 3 commercially available sources (according to the country of origin) of BN and the cross-reactivity between these BN allergens.

METHODS

Sera of patients and source of BN

Sera of 25 patients allergic to BN were obtained after informed consent and stored in aliquots at -80°C until used. These 25 children

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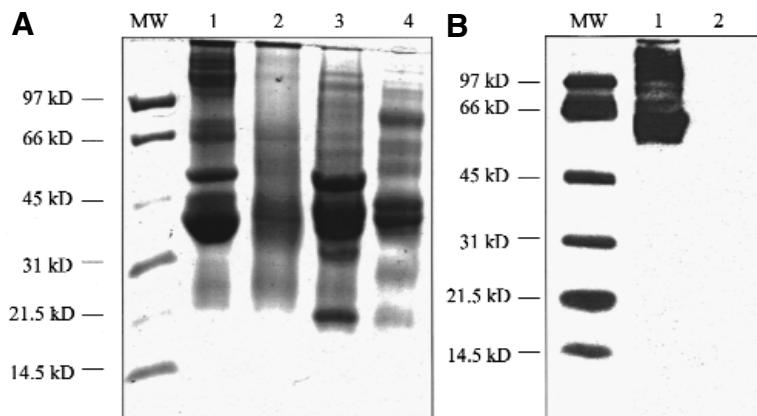


FIG 1. A, SDS (7.5%) protein profiles. Lane 1, Fresh BN from Sarawak (20 μ g/lane); lane 2, commercially available BN from Sarawak BN (20 μ g/lane); lane 3, BN from Indonesia (20 μ g/lane); and lane 4, BN from Thailand (20 μ g/lane). B, IgE immunoblot done with fresh unprocessed BN from Sarawak with allergic sera (patient 1, lane 1) and negative control (lane 2). MW, Molecular weight marker.

are a subset of the cases previously reported.⁶ Commercially available processed BN originating from several regions (Sarawak in Malaysia, Thailand, and Indonesia) was purchased from retailers in Singapore. These represent the main sources consumed by the general population. Fresh unprocessed BN from caves in Sarawak was also obtained.

BN crude extracts

Crude extracts of BN were made by means of aqueous extraction. Feathers in the nests were first manually removed. The nests were then pounded in a mortar, suspended in 1:5 (vol/vol) distilled deionized water, and allowed to elute for 24 hours at 4°C. An aliquot of each extract was boiled for an hour to simulate the way the delicacy is prepared. All supernatants were lyophilized, and the lyophilized powders were stored at -80°C until used.

Detection of BN-specific IgE

Quantification of BN-specific IgE was done by using fluorescence allergosorbent tests (FASTs). Lyophilized crude extracts of boiled commercially available processed BN from Sarawak, Indonesia, and Thailand were reconstituted with FAST (BioWhittaker) coating buffer (at a concentration of 0.1 mg/mL) and used to coat activated wells (BioWhittaker) in accordance with the manufacturer's instructions. Briefly, 100 μ L of the coating solution was added to each well. This was incubated at 37°C for 1 hour and then at room temperature for 18 hours. The wells were then blocked, aspirated, and allowed to dry at 4°C in a dessicator. These wells were then used to detect BN-specific IgE by using IgE FAST Plus Test (BioWhittaker) reagent kits.

FAST inhibition studies

To evaluate cross-reactivity among crude extracts of the 3 boiled commercially available processed BN, pooled positive sera were first separately incubated with increasing concentrations of the following inhibitors: BSA (negative control) or boiled crude extracts of commercially available processed BN from Sarawak, Indonesia, or Thailand. After preabsorption, specific IgE levels were determined by using the FAST assay. Inhibition curves were obtained by plotting the percentage of inhibition against the concentration of inhibitor used.

Protein profiles

Protein profiles of crude extracts of commercially available processed BN from Sarawak and fresh unprocessed BN from

Sarawak were defined by using SDS-PAGE.⁷ SDS gels (7.5%) were run with Mini-PROTEAN II Electrophoresis Cell (BioRad) and stained with Coomassie blue (0.1% Coomassie blue R-250 in 40% methanol and 10% glacial acetic acid).

IgE immunoblots

Because the commercially available BN from Sarawak elicited the highest levels of specific IgE in our patients, further immunochemical characterization was carried out with this type of BN. Our initial experiments with commercially available BN from Sarawak showed few protein bands and smears on SDS-PAGE (Fig 1, A), and IgE immunoblot experiments did not detect any distinct IgE-binding proteins (Fig 1, B). However, because BN-specific IgE was detected in the sera of allergic patients by using FASTs, we hypothesized that commercial processing had possibly resulted in degradation of the proteins while still retaining allergenicity.

We therefore proceeded with experiments with fresh unprocessed BN from Sarawak. SDS-PAGE electrophoresis (7.5%) of crude extracts of fresh unboiled BN from Sarawak in one dimension was carried out under reduced conditions.^{7,8} Electrophoresis onto nitrocellulose (Hybond-C extra, Amersham) was carried out for 2 hours at 150 mA. IgE immunoblotting was carried out according to standard methods.⁹ In brief, membranes were blocked with 3% nonfat milk in 0.05% PBS-Tween (PBST) for 1 hour at room temperature, washed with PBST, and then incubated with sera of patients and control subjects (2 $\times$ dilution with PBST plus 1% nonfat milk) for 18 hours at 4°C. After 6 washes with PBST, the membranes were incubated for 1 hour at room temperature with a biotin-labeled mouse monoclonal anti-human IgE antibody (2 μ g/mL PBST, PharMingen). They were washed 6 times with PBST and incubated for 1 hour at room temperature with a peroxidase-conjugated Extravidin (1:2000 PBST, Sigma). The strips were washed 6 times with PBST and developed with ECL-plus reagent (Amersham).

Two-dimensional electrophoresis

The crude extract of fresh unboiled BN from Sarawak was dissolved in a cocktail of 9 mol/L urea (Bio-Rad Laboratories), 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulphonate (CHAPS; USB, Amersham Pharmacia Biotech AB), 40 mmol/L TRIS (J. T. Baker, Phillipsburg, NJ), and 1 mmol/L phenylmethylsulfonyl fluoride (Sigma Chemical Co). Two-dimensional gel electrophoresis was performed essentially according to the method of Seow et al.⁸ The amount of protein loaded was approximately 100

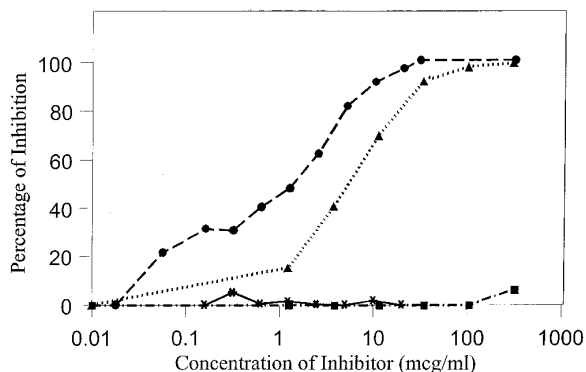


FIG 2. FAST inhibition curves. Solid phase is defined as commercially available processed BN from Sarawak. Liquid phase is defined as pooled positive sera preabsorbed with commercially available processed BN from Sarawak (circles), Thailand (triangles), and Indonesia (squares) or BSA (asterisks, negative control). Levels of BN-specific IgE after preabsorption were determined by means of FAST assay.

µg of protein per gel. The first dimension of IEF was performed on 13-cm pH 4-7 IPG strips (Amersham Pharmacia) at 20°C, with a maximum current setting of 50 µA per strip by 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 per gel at 10°C by using a Bio-Rad Protean II xi electrophoresis unit.

Two-dimensional IgE immunoblots

BN proteins separated by means of 2-dimensional electrophoresis were transferred for 2 hours at 150 mA onto a nitrocellulose (Hybond-C extra, Amersham Pharmacia) membrane. The membrane was used for immunoblotting by using the method described above.

N-terminal amino acid sequencing of identified allergens

Two-dimensional electrophoresis-separated BN proteins were blotted to polyvinylidene difluoride membranes and visualized with Coomassie Brilliant Blue stain. The protein spots of interest were excised, and N-terminal sequencing was obtained by means of automated Edman degradation with an Applied Biosystem 477A Protein Sequencer.

Periodate treatment

The effect of periodate treatment on the IgE-binding capacity of BN allergens was assessed by using positive sera.¹¹ Single nitrocellulose strips with electroblotted BN proteins were oxidized with 0.1 mol/L sodium periodate (100 mmol/L periodic acid in 10 mmol/L sodium acetate, pH 5.0) or sodium acetate buffer alone (control) for 1 hour at room temperature, and the subsequent IgE-binding patterns were compared.

Heat stability studies

To determine the effect of heat, an extract of fresh BN from Sarawak was boiled for known intervals. The effects on protein profiles and specific IgE-binding capacity were assessed by means of 2-dimensional gel electrophoresis and FAST, respectively.

Statistical analysis

The data was collated and analyzed by using SPSS for Windows, version 8.

RESULTS

BN-specific IgE

The levels of BN-specific IgE to the 3 commercially available processed BNs (from Sarawak, Indonesia, and Thailand) were determined in 25 patients allergic to BN (17 male and 8 female patients; average age, 6.12 years; age range, 1-14 years).⁶ The results showed that the BN from Sarawak had the highest number of positive test results (defined as specific IgE >0.75 U/mL or greater than class I reaction) and the highest geometric mean specific IgE level ($P < .001$) when compared with the BN from Thailand and Indonesia (Table I). In contrast, none of the nonallergic control subjects had a positive test result to any of the 3 commercially available processed sources of BN. These data suggest that the BN from Sarawak was the most allergenic.

FAST inhibition studies

The results showed that the Sarawak and Thailand sources were highly cross-reactive (Fig 2). In contrast, the BN from Indonesia showed little allergenic cross-reactivity with the Sarawak source. These findings suggest allergenic heterogeneity among sources of BN.

Protein profiles

The protein profiles of commercially available BN and fresh BN from Sarawak are shown in Fig 1. They have different protein profiles, with the fresh unprocessed BN having more and distinct protein bands. These findings suggest that commercial processing may have reduced the amount of intact protein originally present in the fresh nests.

IgE immunoblots

IgE immunoblots with fresh unprocessed BN from Sarawak identified IgE binding in 17 of 20 patients. All 17 sera showed binding to the putative major allergen, a 66-kd protein, with 8 of 17 also showing binding to a 100-kd protein (Fig 3).⁶ The 2-dimensional IgE immunoblot of the fresh BN from Sarawak showed the presence of isoforms at 66 kd (Fig 4).

Effect of periodate treatment

Periodate treatment of the BNs resulted in the loss of IgE binding on immunoblot studies (Fig 5).

Heat stability studies

The BN proteins, including the 66-kd putative major allergen, degraded with boiling, as shown by progressive loss of molecular weight with increasing intervals of heating (Fig 6). In 2 separate experiments the boiled extract resulted in only modest or no reduction of specific IgE from between 0% and, at most, 26%, as demonstrated by means of FAST, indicating the persistence of IgE-binding capacity despite degradation from boiling.

Putative major allergen

The N-terminal sequences of the 66-kd IgE-binding

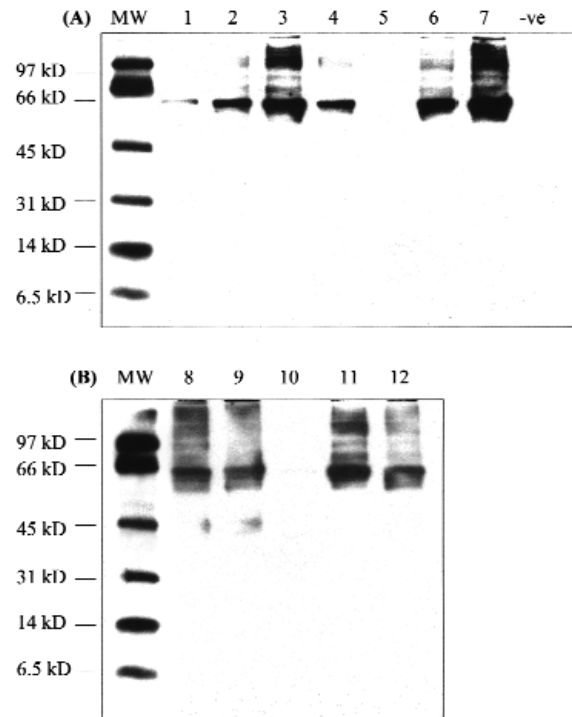


FIG 3. IgE immunoblots with one-dimensional protein profile of fresh BN from Sarawak. Thirty micrograms of protein was loaded to each lane of a 7.5% SDS-PAGE gel separated by means of vertical electrophoresis and electroblotted to nitrocellulose. Blots were blocked with 3% nonfat milk in 0.05% PBST for 1 hour, incubated with sera (2× dilution with PBST + 1% nonfat milk) for 18 hours at 4°C, and developed with ECL-plus reagent (Amersham). Lanes 1 to 12, Immunoblots done by using positive sera from 12 allergic patients; -ve, Negative control; MW, molecular weight marker.

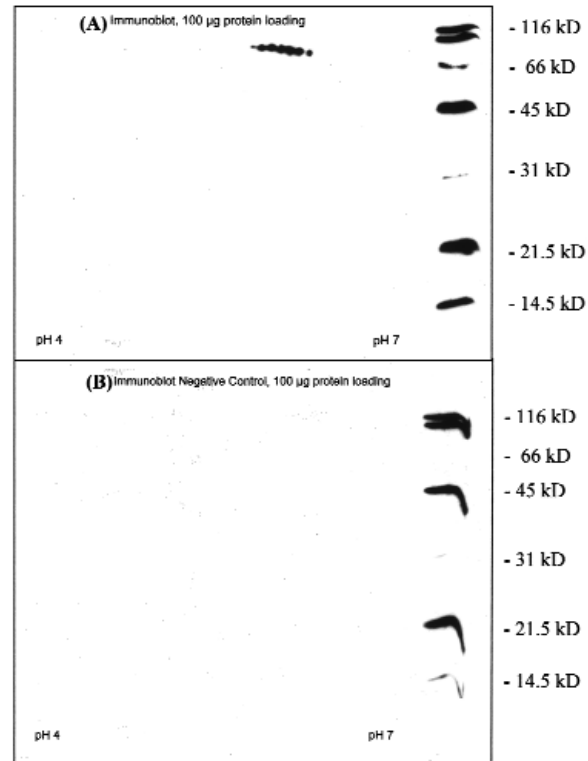


FIG 4. IgE immunoblots by using 2-dimensional protein profile of fresh BN from Sarawak. Protein load was approximately 100 µg of protein per gel. The first dimension of IEF was performed on 13-cm pH 4-7 IPG strips (Amersham Pharmacia). Second dimension separation was carried out by using 13% SDS-PAGE. The separated proteins were electroblotted to nitrocellulose, blocked with 3% nonfat milk in 0.05% PBST for 1 hour, incubated with sera (5× dilution in PBST + 1% nonfat milk) for 18 hours at 4°C, and developed with ECL-plus reagent (Amersham). **A**, Immunoblot done with serum from patient 1 (see Fig 2). **B**, Immunoblot done with negative control.

TABLE I. FAST results in patients with BN allergy and nonallergic control subjects

Allergen	Geometric mean of BN-specific IgE levels in patients with BN allergy, range (IU/mL)	No. with positive FAST results*		P value†
		Patients with BN allergy	Control subjects	
Sarawak BN	1.94 (0.10-59.67)‡	18/25	0/36	<.001
Indonesia BN	0.17 (0.05-14.92)	1/25	0/36	.4104
Thailand BN	0.28 (0.06-26.25)	4/25	0/36	.024

*Positive FAST test result was defined as specific IgE levels of greater than 0.75 IU/mL (corresponding to FAST class 2).

†P values were obtained by using the Fisher exact test (allergic patients vs control subjects).

‡P values of less than .0001 with the Mann-Whitney test (comparing geometric means of BN from Sarawak vs BN from Indonesia and BN from Sarawak vs BN from Thailand).

protein band of the BN allergens from the Sarawak source was obtained (Table II) and was found to show homology to a domain of an ovoinhibitor precursor in chicken (SWISS-PROT accession No. P10184).

DISCUSSION

Our interest in BN allergy was sparked by our observation of children hospitalized for anaphylaxis after

ingestion of edible BNs. Indeed, we found it to be the most common cause of hospitalization for food-induced anaphylaxis in children in our community.⁵ Its prevalence surpassed well-defined food allergens (eg, cow's milk or egg allergy in younger children and peanut or crustacean seafood in older children). Food-induced anaphylaxis is a potentially lethal reaction. Deaths are related to undiagnosed allergy or to the unwitting consumption of the allergen. Prevention of such events requires

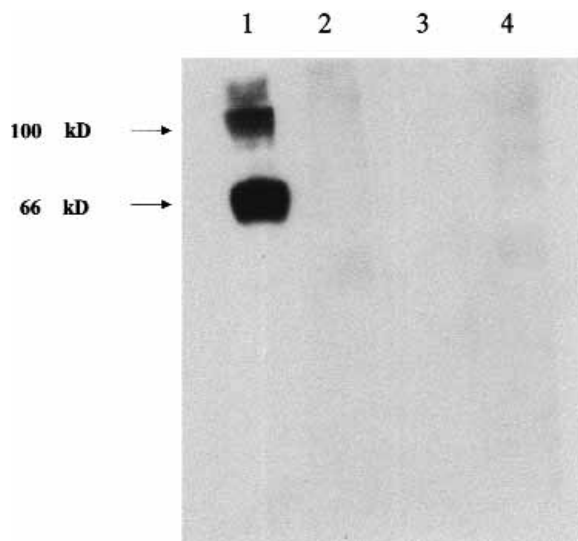


FIG 5. Effect of periodate treatment of BN on IgE immunoblots. Single nitrocellulose strips with electroblotted BN proteins were oxidized with 0.1 mol/L sodium periodate (100 mmol/L periodic acid in 10 mmol/L sodium acetate, pH 5.0) or sodium acetate buffer alone (control) for 1 hour at room temperature. The blots were subsequently blocked, incubated with sera (5× dilution in PBST plus 1% nonfat milk) for 18 hours at 4°C, and developed with ECL-plus reagent (Amersham). The IgE-binding patterns are shown. *Lane 1*, Untreated blot with positive sera; *lane 2*, untreated blot with negative control; *lane 3*, treated blot with positive sera; and *lane 4*, treated blot with negative control.

accurate identification of the food allergen, understanding of its structure, and study of its possible cross-reactivity with other food or inhalant allergens. Given that BNs are widely consumed by populations (particularly the ethnic Chinese) worldwide, it is therefore important to highlight their potential to induce anaphylaxis and define their allergens.

Some of the world's major producers of BNs are Malaysia (Sarawak), Thailand, and Indonesia. BNs in these countries are harvested, processed, and exported worldwide for commercial sale. These commercially available processed nests are similar in appearance and are identified only by their country of origin. They are not identified by the species of swiftlets that produce them. The 3 sources of commercially available processed BN evaluated in this study appear to be heterogeneous, as made evident by FAST results and cross-reactivity studies. These differences may be related to the species of swiftlets that produced them, but further work is needed to confirm this.

Of the 3 commercially available processed BN sources studied, the BN from Sarawak gave the highest prevalence of positive test results for BN-specific IgE and induced the highest geometric mean specific IgE level in patients with BN allergy. It was for these reasons that we proceeded to evaluate the BN allergens in the Sarawak source. Our initial IgE immunoblots with commercially available processed BN from Sarawak were unsuccessful in identifying distinct IgE-binding proteins. Given that we

could detect Sarawak BN-specific IgE by means of FAST, we hypothesize that commercial processing had reduced the amount of intact protein allergens to a quantity still sufficient to elicit an allergic reaction but insufficient to detect by means of IgE immunoblot. We tested the fresh unprocessed BN from Sarawak for allergenicity. FAST was done on 6 patients (patients 7, 18, 19, 21, 24, and 25). Five of 6 had reactions greater than class I, and 3 of 6 had reactions greater than or equal to class II, where specific IgE was greater than 0.75 IU/L. In contrast, sera from 6 control subjects not allergic to BNs were negative. By using fresh BN extracts from Sarawak, IgE blots showed distinct IgE-binding proteins and a putative major 66-kD allergen. Because this protein band was not detected on SDS-PAGE in the commercial source of BN from Sarawak, it is possible that commercial preparation may have (1) reduced the amounts of native allergens to such a small amount that it is undetectable on immunoblot but sufficient enough to trigger an allergic reaction *in vivo*, (2) resulted in degradation of the intact protein allergen to smaller molecules that still retain its allergenic epitope (because commercial processing also includes oven baking), (3) or both. The latter is suggested by heat treatment (boiling) of the extracts of the fresh BNs, which showed progressive degradation (reduction on molecular weight) of the 66-kD protein (Fig 6).

Because BN is composed of the saliva of swiftlets, its allergens and their isoforms are likely to be salivary proteins. The only other salivary proteins (although not ingested) that are known to cause allergic reactions are those of the mosquito. Mosquito bites are well known to cause local reactions, although more rarely, angioedema, asthma, and anaphylaxis have been described.¹⁰ Immunoblotting of mosquito proteins have verified the presence of multiple IgE-binding proteins,^{11,12} although the exact nature of these proteins has not been studied. Our preliminary data on N-terminal amino acid sequencing of the 66-kD protein in BN from Sarawak suggests that this protein shows homology to an ovoinhibitor precursor in chicken. We are currently pursuing further studies to determine the exact nature of this protein.

The loss of IgE-binding ability after periodate treatment suggests that the carbohydrate moiety may contribute to the allergenic epitopes. This phenomenon has also been known to occur with the allergens of celery,¹³ where the α -1,3-fucose carbohydrate moiety is a key component of the IgE-binding epitope or epitopes, and these structural components are very resistant to boiling and cooking.

Most proteinaceous food allergens contain epitopes resistant to cooking and digestion. This phenomenon is also present in BN allergy. Historically, the fact that the anaphylactic reactions were triggered by the consumption of boiled BN suggested that the allergenic epitopes are likely to be heat resistant. This was proven experimentally because the IgE binding, as assessed with FAST, was little affected by boiling the extracts for prolonged periods.

Because BN is processed before commercial sale, we also considered the possibility that the allergic reactions

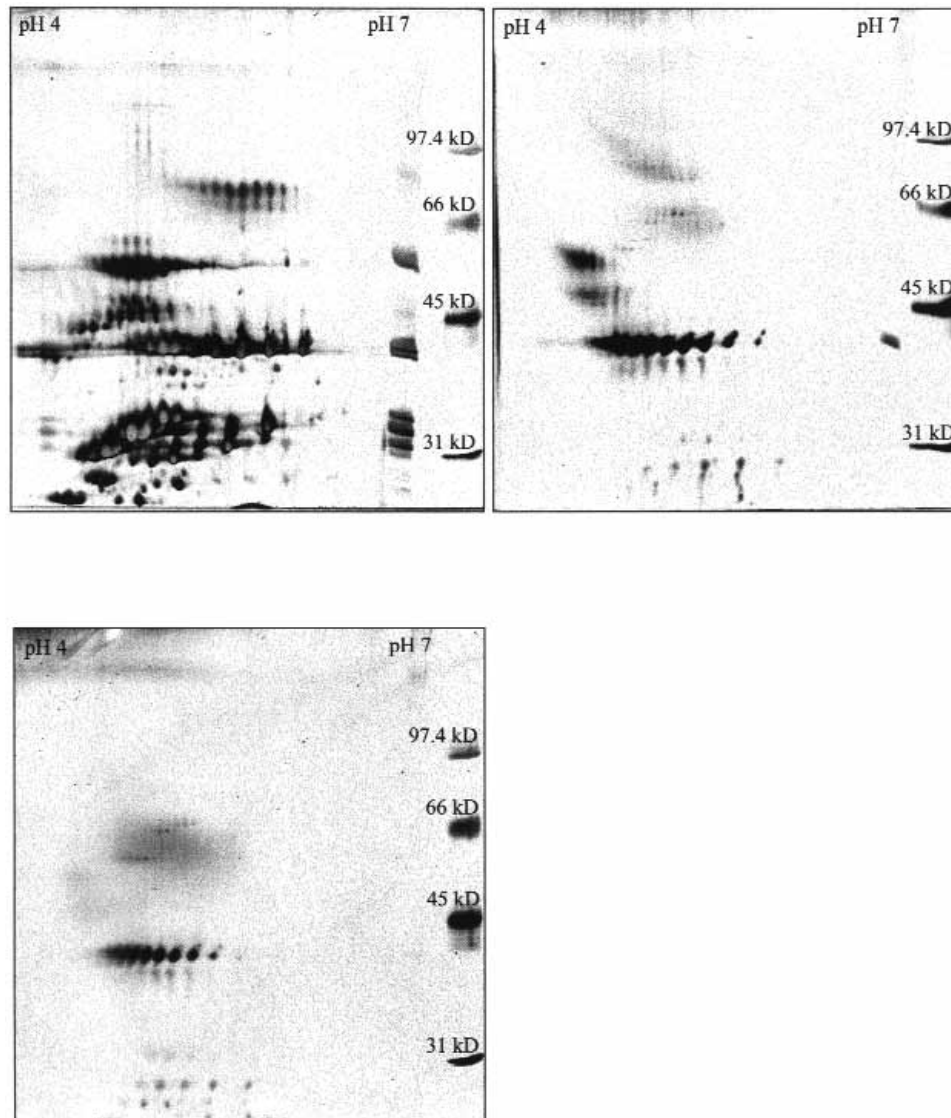


FIG 6. Two-dimensional SDS-PAGE gel showing protein profiles of fresh BN from Sarawak and the effect of heat treatment. **A**, Fresh unboiled BN from Sarawak (187 μ g of protein per gel); **B**, fresh BN from Sarawak boiled for 10 minutes (148 μ g of protein per gel); and **C**, fresh BN from Sarawak boiled 30 minutes (166 μ g of protein per gel).

TABLE II. Partial N-terminal sequence of the 66-kd protein in fresh BN from Sarawak

Protein	Peptide sequence	Identity (%)	Similarity (%)
66-kd protein (fresh BN from Sarawak)	VEVDCSQYSSDVTKDGTVRV * * * *	55	75
Ovoinhibitor	IEVNCS LYASGIGKDGTSWV		

were caused by additives or contaminants. Several candidates were considered: gelatin, feathers, or proteins in crude oil. Gelatin has been known to be surreptitiously added in the final steps of processing, so as to increase the bulk of the edible nests.¹ None of our patients had a history of gelatin allergy, which has rarely been reported.¹⁴ Although feather allergens are generally considered

to be inhalant rather than food allergens,¹⁵ there has been documentation of cross-reactivity between feather and egg protein allergens.¹⁶ Skin prick tests with extracts of porcine and bovine gelatin and swiftlet feathers on 9 of our allergic patients showed negative responses, indicating absence of sensitization to these allergens (data not shown).

In conclusion, allergens of BN from difference sources are heterogeneous. The immunochemical characteristics of the Sarawak source of BN are described in this study. Preliminary data suggests that the putative major allergen shows homology to a domain of an ovoinhibitor precursor in chicken (SWISS-PROT accession No. P10184). Further work is required to identify these allergens, which are likely to be salivary proteins of swiftlets.

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