

# Preparation and Characterization of Silverfish (*Lepisma saccharina*) Extract and Identification of Allergenic Components

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## Key Words

Insect allergy · Indoor allergens · Extract characterization

## Abstract

**Background:** Airborne insect antigens represent important aeroallergens which have been widely investigated. Although it has been demonstrated that house dust contains significant silverfish (*Lepisma saccharina*) levels, none of the extracts obtained so far has been extensively characterized. Thus, we have prepared and characterized a silverfish extract and investigated its IgE-reactive components by testing the reactivity of sera from patients allergic to inhalant insect allergens. **Methods:** The extract from silverfish insect bodies was prepared by homogenizing frozen silverfish in Tris-HCl buffer. The soluble material (Sup) was filtered and the insoluble material (Ppt) was resuspended in 100 mM Tris pH 10.6. The two fractions were characterized by biochemical and immunochemical methods. IgE reactivity was investigated on both fractions before and after periodate treatment. **Results:** Protein content and total carbohydrates was 2 and 3% w/w for Sup and 1 and 0.3% w/w for Ppt. The SDS-PAGE profile of the two fractions showed a different pattern in the MW range of 5–175 kD. Sup and Ppt, probed with allergic sera, showed a complex pattern of IgE reac-

tivity. When periodate-treated fractions were tested, IgE reactivity was either completely abrogated, reduced or not affected, depending on the allergic serum employed. **Conclusions:** The results obtained indicate that the classic aqueous-extraction procedures that have been used up to now for other insects might not be completely satisfactory, since several allergenic components are not soluble at the normally used pH. We developed a dedicated extraction procedure allowing the detection of a certain degree of reactivity in sera negative to allergens extracted following classic procedures.

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## Introduction

Insects and insect-derived materials have been recognized as risk factors for sensitization and subsequent elicitation of allergic rhinitis and allergic bronchial asthma [1–5]. Contact with insects or insect materials can lead to allergic sensitization as a consequence of either natural, domestic, occupational, or hobby-related exposure. Consequently, insects can be considered indoor as well as outdoor inhalant allergens.

In the early 1930s, the role of insects in allergies was essentially investigated in a series of isolated cases in

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which the inhalant allergy was attributed to insects present in outdoor environments or in work place. In these studies, a variety of insects, such as mayflies [6], caddis flies [7], chironomid midges [8], mushroom flies [9], moths and butterflies [10], were identified as possible causes of insect allergies. Parlato [10] and Balyeat et al. [11] compared the distribution of the common pollens and fly emanations using morphologic methods. The results showed that the amount of insect emanations during the June to September period closely corresponded to the amount of pollen.

More recently, Kino et al. [12], using an immunochemical method previously developed by Agarwal et al. [13], confirmed that in outdoor air the concentration of airborne insect-related allergenic particulate <10 µm in diameter falls in the same range as that reported for pollen allergens [14]. Moreover, significant concentrations of insect allergens have also been measured in indoor air [15].

During the last decades, insects other than those known as allergenic, were investigated for their potential role in inducing and triggering an IgE immune response [16, 17]. Among these, the silverfish, an insect belonging to the Thysanura order, appeared to be of particular interest [16, 18–20]. Its importance significantly increased when Witteman et al. [19] demonstrated that dust samples contain significantly high silverfish levels, even in houses where the inhabitants were unaware of its presence.

Although the assay developed by Witteman et al. provided an important tool for establishing the presence of silverfish material in various places, silverfish allergens have been neither characterized nor purified, and no silverfish extract for diagnosis of allergic disease is commercially available.

In order to address the issue of extract characterization, we developed an extraction procedure yielding a silverfish extract of satisfactory quality, which was analyzed by means of immunochemical assays to identify the various components and investigate their allergenic importance.

## Materials and Methods

### Human Sera

Two groups of subjects were enrolled in the study: 202 randomly selected subjects and 50 patients suffering from insect allergic sensitization (50 subjects). Ten of the 50 patients presented positive skin test and RAST to at least one of the five following allergens: house fly (*Musca domestica*), cockroach (*Blattella germanica*), spider (*Tegenaria*

*domestica*), clothes mite (*Tineola bisselliella*), *Ephestia cautella*. The remaining 40 patients were tested only by skin test with 3 allergens: *B. germanica*, blackfly and mosquito whole body (*Aedes communis*) and only those having at least one positive skin test were included in the study. A nasal provocation test to the most relevant allergens was positive for all 40 patients. In general, positivity to several allergens was observed in the group of patients, indicating insect panallergy.

It should be pointed out that all the patients had been tested with a broad panel of allergens including pollens (grasses, *Parietaria judaica*, *Olea europea*, *Cupressus sempervirens*), molds (*Alternaria alternata*), cat dander and mites (*Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*). Specifically, almost all of the patients were positive to a skin or RAST test to mites.

A skin prick test was performed with the Dome Hollister lancet using extracts in a 1/20 solution (wt/vol). We adopted the following scores: 3+ was assigned to the wheal and erythema of histamine (10 mg for 1 ml prick test; 1% weight volume); 4+ was assigned to a reaction greater than that of histamine; 2+ was assigned to a 75% reaction and 1+ was assigned to a reaction half the size of the histamine reaction. The test was considered positive only for patients showing a 2+–4+ skin reactivity. Informed consent was obtained from all patients.

### Lepisma saccharina Extract Preparation

Silverfish (*L. saccharina*) were collected between late March and late October in different houses in Rome. The insects were killed by freezing and stored at –80°C until use. Sixty milligrams of silverfish insects (3 bodies) were homogenized by 10-min strokes of a glass-teflon homogenizer in 1 ml of 100 mM Tris-HCl pH 8. The homogenate was then extracted by slow stirring at +4°C for 10 min and centrifuged at +4°C for 10 min. The supernatant (Sup) was filtered through a 0.22-µm Millipore filter. The resultant pellet (Ppt) was resuspended in 300 µl of 100 mM Tris pH 10.6. The two fractions were aliquoted and stored at –20°C. Protein and carbohydrate contents were measured according to the methods of Bradford [21] and Dubois et al. [22], respectively.

### Polyacrylamide Gel Electrophoresis

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransfer were carried out as previously described in a mini-system (Bio-Rad, Richmond, Calif., USA) [23, 24]. Ppt and Sup (10 µg protein/well) were reduced by adding 50% v/v 2-mercaptoethanol before application to the 15% w/v polyacrylamide gel. The gel was stained with 0.05% Coomassie Brilliant Blue in water:methanol:acetic acid (50:40:10) for protein detection.

### IgE Immunoblotting

Immunoblotting was performed as described by Barletta et al. [25] in a Bio-Rad apparatus after SDS-PAGE separation. The blotted nitrocellulose strips were incubated overnight at room temperature with 1 ml of individual serum diluted 1:2 in phosphate-buffered saline-Tween 20, 0.05% v/v, pH 7.2 (PBS-Tween), washed 5 times with PBS-Tween, and then incubated overnight with <sup>125</sup>I-labeled anti-human IgE (Bio-Allergy) diluted in PBS-Tween. Strips were developed by exposing the blots to X-ray film (Kodak Diagnostic Film X-Omat AR; Eastman Kodak Company, Rochester, N.Y., USA). Strips incubated with PBS-Tween instead of serum samples were used as negative controls.

### Lectin Blotting

The specific binding of lectins to the carbohydrate moieties present on the components of Sup and Ppt was studied by using the Vectastain Lectin Kit (Vector Laboratories, Burlingame, Calif., USA), according to the manufacturer's instructions. After SDS-PAGE and electroblotting, the nitrocellulose-blotted strips were incubated for 1 h at room temperature with a final concentration of 15 µg/ml of the following biotinylated lectins: *Ricinus communis* (RCA), *Pisum sativum* (PSA), *Sophora japonica* (SJA), *Triticum vulgare* (WGA), *Glycine max* (SBA), *Arachis hypogaea* (PNA), *Griffonia* (GSL), *Lens culinaris* (LCA), *Phaseolus vulgaris* erythroagglutinin (PHA-E), *Canavalia ensiformis* (Con A), *Dolichos biflorus* (DBA), *Ulex europaeus* (UEA), *P. vulgaris* leucoagglutinin (PHA-L). After washing 5 times with PBS-Tween, strips were incubated with Vectastain ABC-Immunoperoxidase Reagent for 30 min at room temperature. The colorimetric reaction was performed with 3,3-diaminobenzidine according to Sigma instructions.

### ELISA for Specific IgE Detection

ELISA experiments were performed according to the procedure developed in our laboratory [26] by using Sup and Ppt fractions as antigens at an optimal concentration of 20 µg/ml established on the basis of preliminary experiments. Individual sera were tested at 1:5 dilution and IgE binding was detected by a peroxidase-labeled rabbit antibody to human IgE (KPL, Gaithersburg, Md., USA). A mean value + 3 SD of the optical density values, obtained with 6 normal subjects, was regarded as the negative cut-off.

### Periodate Treatment

Sup and Ppt were subjected to NaIO<sub>4</sub> oxidization after absorption on ELISA plates according to the method described by Woodward et al. [27]. Antigens adsorbed on the plate were exposed to 10 mM NaIO<sub>4</sub> for 1 h at room temperature in the dark, whereas control wells (mock) were treated with the buffer only. Control and experimental wells were incubated with 50 mM NaBH<sub>4</sub> PBS for 30 min. The test was then continued as described above.

### Dot Immunoblot Test

A nitrocellulose membrane was spotted with 2 µl of diluted antigen (Sup and Ppt) at a concentration of 1 µg/µl and air-dried. Detection of IgE binding was the same as in the IgE immunoblotting.

## Results

### Production of Silverfish Extract

Preliminary extraction experiments, carried out in an aqueous buffer of about pH 7, suggested that the resulting silverfish extracts could lack important allergenic components when tested for IgE reactivity after immunoblotting (data not shown). Thus, a specific procedure was developed leading to two fractions, one obtained from an aqueous extraction in 100 mM Tris pH 8 (Sup) and the other from the insoluble material resuspended in 100 mM Tris pH 10.6 (Ppt).

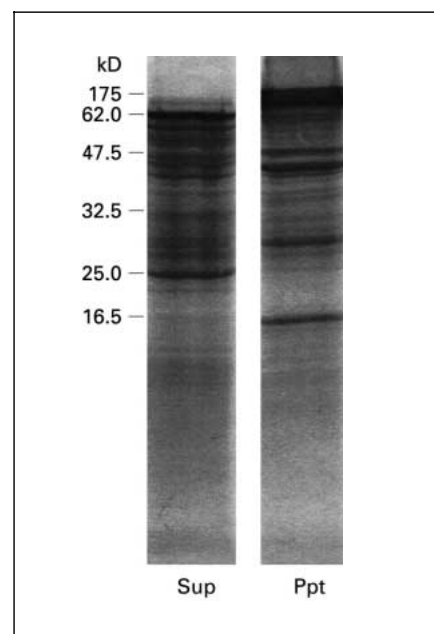


Fig. 1. Two fractions of the silverfish extract after separation by SDS-PAGE in reducing conditions and Coomassie Brilliant Blue staining.

A protein yield of about 3% (w/w) for both fractions was obtained, whereas total carbohydrates represented 1 and 0.3% w/w of the Sup and Ppt, respectively.

The two fractions stained by Coomassie Brilliant Blue showed a different pattern in a broad MW range from 5 to over 175 kD (fig. 1). The presence of some high MW bands and of a 14-kD component was restricted to the Ppt fraction only. Common components were detected in both fractions but with differing intensities.

### Lectin Binding

The presence of carbohydrate moieties in the two fractions of the extract was studied by testing the binding of the lectins listed in Material and Methods on the blotted antigens. Con A, LCA and PSA lectins showed a great intensity of reaction in the MW range of 97.4–40 kD in both fractions and with comparable patterns, thus indicating that most of the Sup (fig. 2a) and Ppt (fig. 2b) glycoproteins contain  $\alpha$ -mannose residues. DBA lectin, specific for  $\alpha$ -N-acetylgalactosamine, showed additional components in the MW range of 20 kD in both fractions. The other lectins showed a weak reactivity essentially restricted to one component with MW of about 66 kD. SJA did not react with any of the individual components.

Table 1. Prevalence of silverfish sensitivity by ELISA, dot blot and immunoblotting in the two groups studied

| Group                      | n   | ELISA     |           | Dot blot |        |
|----------------------------|-----|-----------|-----------|----------|--------|
|                            |     | Ppt       | Sup       | Ppt      | Sup    |
| Randomly selected subjects | 202 | 12 (5.9%) | 11 (5.4%) | n.d.     | n.d.   |
| Patients                   | 50  | 9 (18%)   | 15 (30%)  | 17 (34%) | 4 (8%) |

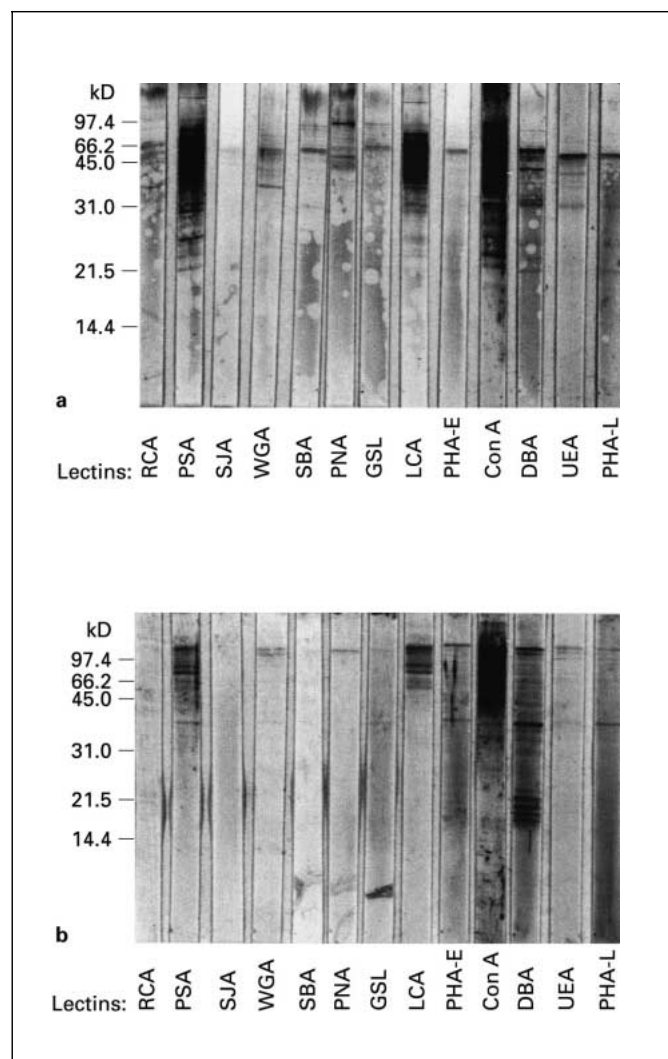


Fig. 2. Lectin-binding patterns of Sup (a) and Ppt (b) after blotting.

#### Prevalence of IgE Specific Reactivity by Solid-Phase Immunoassays

Two groups of subjects were enrolled in the study, one consisting of 202 randomly selected subjects, and the other of 50 patients suffering from insect respiratory allergy.

Sera belonging to the random population were screened by ELISA: 11 (5.4%) and 12 (5.9%) of the 202 subjects recognized Sup and Ppt fractions, respectively. However, 15 of the 202 subjects (7.4%) recognized at least one fraction of silverfish extract. The allergic population was also screened by ELISA, and the positivity in this group was 30 and 18% for Sup and Ppt, respectively (table 1).

To evaluate the influence of the solid phase and of the technique itself on the number of positive subjects, sera from the group of allergic subjects were also tested by dot blot. The results indicated a higher reactivity with Ppt (34% dot blot vs. 18% ELISA) and a lower reactivity with Sup (8% dot blot vs. 30% ELISA). Taken together, these data support that allergenic molecules present in the two fractions behave differently according to the coupling conditions and testing procedures.

#### IgE Binding on Silverfish Extract by Immunoblotting

To better characterize IgE reactivity to the two fractions at the level of individual components, a panel of 19 sera reactive in IgE immunoblotting was selected.

Frequency of the IgE recognition of the silverfish extract components was calculated on the 19 sera positive in immunoblotting (fig. 3a, b). The Sup fraction (fig. 3a) was characterized by 17 bands with MWs ranging from about 100 to 6.5 kD. The highest frequency of recognition (42%) was reached with a 29-kD component. Other components were similarly reactive, whereas four components were reactive only with 5% of the sera tested. The Ppt fraction (fig. 3b) contains 21 allergenic components ranging from about 100 to 19 kD. A group of components with an MW close to 100 kD was recognized by the highest proportion of sera (13/19, corresponding to 68%), followed by bands with MW of 83 and 31–35 kD (32 and 21%, respectively). The remaining components were recognized with variable frequencies but none exceeding 10%. The component with an MW of 31–35 kD showed the greatest intensity of reaction (data not shown).

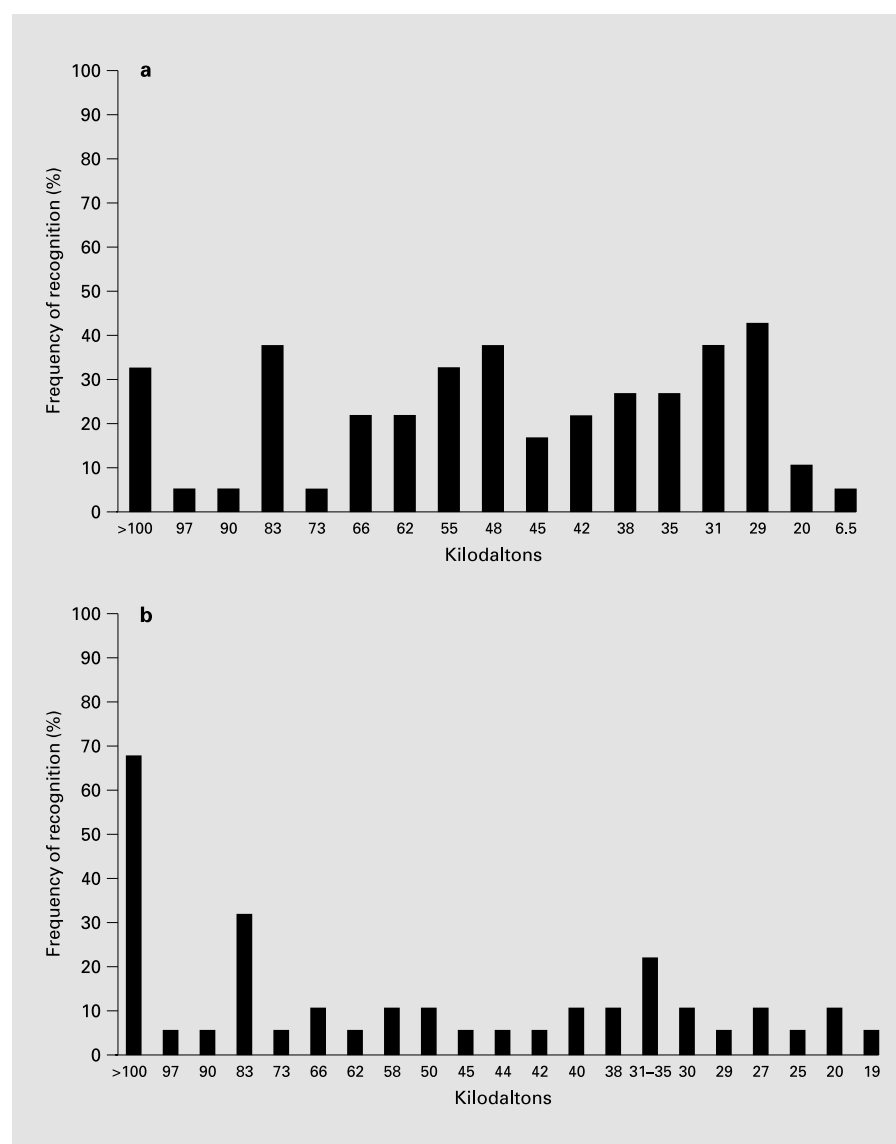


Fig. 3. Molecular weight and frequency of IgE recognition of components detected in immunoblotting in Sup (a) and Ppt (b) fractions.

#### *IgE Reactivity after Periodate Treatment*

The involvement of carbohydrate determinants in IgE reactivity to both fractions was evaluated by ELISA performed on deglycosylated Ppt and Sup with a panel of sera from allergic subjects. Table 2 reports representative results which indicate that IgE reactivity could be completely abrogated (serum C), reduced (serum B) or not affected at all (serum A), depending on the allergic serum employed.

Table 2. IgE reactivity from 3 representative allergic subjects to the Ppt and Sup after periodate treatment

| Serum | Ppt       |      |      | Sup       |      |      |
|-------|-----------|------|------|-----------|------|------|
|       | untreated | deg  | mock | untreated | deg  | mock |
| A     | 1.47      | 1.42 | 1.36 | 1.35      | 1.30 | 1.28 |
| B     | 1.31      | 0.81 | 1.29 | 1.12      | 0.02 | 1.05 |
| C     | 1.15      | 0.01 | 1.00 | 0.68      | 0.05 | 0.72 |

The results are expressed in optical densities (OD<sub>490</sub>).

deg = Experimental wells exposed to sodium metaperiodate; mock = control wells treated as 'deg' but without sodium metaperiodate.

## Discussion

Increasing evidence indicates that insects and insect-derived materials represent a potentially significant source of both indoor and outdoor inhalant allergens.

Among indoor insects, great attention has been given to largely diffused insects such as cockroaches. An allergy to cockroaches has been recognized as an important cause of asthma and other allergic diseases in many regions of the world, and cockroaches hypersensitivity is closely related to the rate of household infestation [28, 29].

However, Witteman et al. [19] and Aalberse [20] demonstrated that house dust contains significant level of silverfish antigens and reported that silverfish is also common in Dutch indoor environments.

Silverfish (*L. saccharina*) is the most primitive living insect, and represents a descendent of the ancestral wingless insects. They are 3–12 mm long, have three tail feelers and are covered with shiny scales. They shun light and need a humid environment and their diet consists of carbohydrate materials such as paper and book-binding glue, crumbs of bread and flour. Because of these features, silverfish find an optimal habitat both in dwellings and work places, and unlike cockroaches, their presence can pass unnoticed.

Identification of optimal extraction conditions and characterization of allergenic extracts are the first steps to obtain an effective allergen preparation suitable for diagnosis and therapy, and will be useful as a reference preparation for assessing silverfish exposure in different indoor environments.

Several different extraction methods to obtain insect allergenic extracts have been described in the literature [18, 30–33], confirming that extraction conditions can affect the overall quality of allergenic proteins [34].

This study describes the preparation and characterization of an extract from *L. saccharina* insect bodies and the identification of allergenic components. In preliminary experiments, the optimal extraction protocol was established on the basis of IgE reactivity after immunoblotting. We also took into account the insoluble fraction obtained after the centrifugation step of a classic aqueous extraction. The two fractions did not substantially differ in terms of protein content and showed a complex electrophoretic pattern. Some components of the same MW were common to both Sup and Ppt, though with different intensity, whereas other bands were peculiar to only one fraction.

Both Sup and Ppt were also characterized by lectin blotting analysis on SDS-PAGE-separated fractions. The

results suggest that most of the fractions' components contain glycosidic residues, mainly  $\alpha$ -mannose and N-acetylglucosamine. The role of carbohydrate moieties in the binding of patients' IgE was further investigated by ELISA before and after periodate treatment. These results indicated that several sera showed a reduced IgE reactivity after periodate treatment. These data should be taken into account when evaluating the widespread cross-reactivity at the IgE level among different arthropods.

To evaluate the prevalence of silverfish sensitization, two groups of subjects, one consisting of 202 randomly selected subjects and the other of 50 insect-allergic patients, were screened using both fractions. The screening of the first group was carried out only by ELISA due to the limited volume of serum available. Twelve out of 202 subjects (5.9%) were positive to Ppt fraction, whereas 11 (5.4%) were positive to Sup fraction. Altogether, 15 sera out of 202 (7.4%) recognized at least one fraction of silverfish extract, with 4 subjects (2.0%) specifically reacting to Ppt (insoluble components) only. This finding suggests that silverfish could represent a significant source of indoor inhalant allergens.

In the group of patients, the percentage of positivity in ELISA to Sup fraction of silverfish (30%) is in line with that previously obtained by other authors [16], thus suggesting that our Sup is comparable to other aqueous extracts. On the other hand, the Ppt fraction showed lower ELISA binding as compared to Sup, but gave a higher percentage of positivity in dot blot (34%). Taken together, these results suggest that, besides extraction conditions, coupling conditions to the solid phase might represent a pivotal issue to be considered when evaluating the specific IgE binding in a screening study. On the other hand, the difference in anti-IgE antibodies used in the two assays could also affect the different recognition frequency obtained.

The analysis of the immunoblotting reactivity with the various Sup and Ppt components revealed that a few of them, with apparently the same MW, are recognized in both fractions by some distinct sera, thus suggesting the existence either of identical molecules present in both fractions or of different allergens with almost identical MW. Both conclusions are supported by the reactivity pattern of distinct sera.

The higher frequency of reactivity in the Sup fraction is associated with a band of 29 kD, though many other components are frequently recognized by sera, thus indicating a wide reactivity towards most of the allergenic components of the extract.

On the other hand, Ppt fraction contains only few of the most frequently-recognized components (>100, 83 and 31–35 kD), with all the remaining components being reactive only in fewer patients.

An in-depth evaluation of the restricted reactivity of the Ppt fraction indicated a proportion of sera (21%) recognizing a diffuse and intense component with an MW of 31–35 kD present only in the Ppt fraction. Silverfish tropomyosin could be such a component, since the same sera showed an IgE binding in immunoblotting with shrimp tropomyosin, purified according to the method of Smillie [35] (data not shown). Although tropomyosin has been identified as the major allergen of edible invertebrates, the low frequency of its recognition by our sera from insect-allergic patients suggests that it is not a major allergen in inhalant invertebrates. On the other hand, as tropomyosin has been recognized as an important invertebrate panallergen [36, 37], silverfish tropomyosin could also be regarded as a panallergen molecule. Further studies are required to elucidate this aspect better. Recombinant tropomyosin from silverfish, recently cloned in our laboratory, might be an important tool to clarify this point (manuscript in preparation).

Another group of components showing a molecular weight close to 100 kD is recognized with high frequency in the Ppt fraction. Recently, Tsai et al. [38, 39] have characterized and cloned a novel mite allergen with MW of 98 kD showing a greater than 80% IgE reactivity with sera from patients allergic to mites. Protein sequence analysis and alignment confirmed that the deduced polypeptide is

a mite paramyosin. Taken together, these data suggest that the component identified with higher frequency in the Ppt fraction could be silverfish paramyosin. Further studies are required to evaluate this possibility.

Data reported in the present study confirm that silverfish extract can be recognized by sera from subjects allergic to several household insects, suggesting that either there is a general cross-reactivity among household insects (panallergy) or that silverfish has gone unnoticed in the house of these patients. Moreover, our findings indicate that the classic aqueous extraction procedure is not completely satisfactory in our hands, since important allergenic components are lost at a pH close to neutrality. The insoluble allergenic material, which is normally discarded, might strongly influence the quality of the diagnosis. In fact, an important panallergen, whose cross reactivity appears to play an important role within invertebrates, would be missed. Equal attention should be given to the presence of paramyosin, which, similarly, does not appear to be readily extractable in classic aqueous buffer. Due to common features between silverfish and other arthropods, the issue of extraction conditions should be extended to commercially available extracts prepared from allergen sources such as cockroaches and mites.

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