

Biochemical and Molecular Biological Aspects of Silverfish Allergens

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Abstract: Insects and insect-derived materials have been implicated as a risk factor for sensitization and subsequent elicitation of allergic rhinitis and allergic bronchial asthma. 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. Among these, the silverfish, an insect belonging to the Thysanura order, appeared to be of particular interest. Silverfish (*Lepisma 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 finds an optimal habitat both in dwellings and workplaces and in spite of its antiquity, silverfish has succeeded in exploiting the new opportunity created by man.

Although its importance significantly increased when it has been demonstrated that house dust contains significant silverfish levels even in houses where the inhabitants were unaware of its presence, no silverfish extract for diagnosis of allergic diseases is commercially available yet.

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.

It has been cloned and characterized a silverfish tropomyosin, named Lep s 1, which represents the first allergen identified in silverfish extract and can be regarded as a molecule cross-reactive among inhalant and edible invertebrates allergenic sources. rLep s 1 displayed biological activity, suggesting that it could be regarded as a useful tool to study the role of silverfish tropomyosin in the sensitization to invertebrate allergenic sources.

Keywords: Insect allergy, indoor allergens, silverfish, characterization, cross-reactivity, tropomyosin.

INTRODUCTION

The inhalant allergy to insects and insect-derived materials has been documented since the early 1930s. The body components of insects can become inhalant allergens, such as shed hairs, scales, excreta and bits of disintegrated bodies, causing allergic rhino-conjunctivitis and asthma. Contact with insects or insect materials can lead to allergic sensitization as a consequence of natural, occupational, domestic, or hobby-related exposure. Consequently, insects can be considered indoor as well as outdoor inhalant allergens [1-2].

During the last decades, other insects besides those already known to be allergenic were investigated for their potential role in either inducing or triggering IgE immune response [1-2]. Among these, the silverfish, an insect belonging to the Thysanura order, appeared of particular interest [3]. It is 3–12 mm long, has three tail feelers and is covered with shiny scales (Fig. 1). In spite of its antiquity, silverfish has succeeded in exploiting the new opportunity created by man and it is almost exclusively associated with human houses, stables, outhouses and so on. Silverfish is not easily seen by dwellers because it is nocturnal and can run very

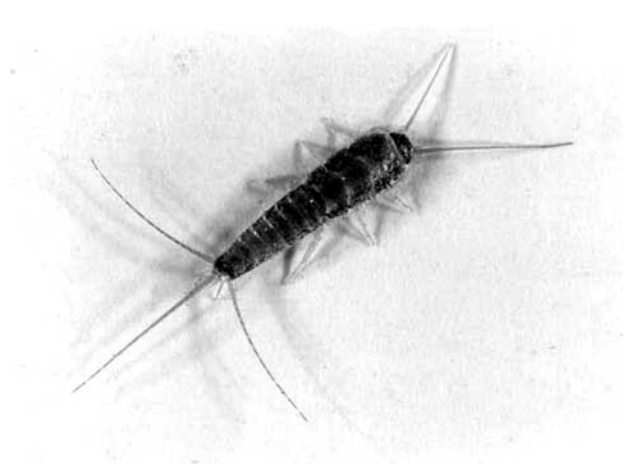


Figure 1.

swiftly. However, it is considered a pest, or at least a nuisance, by homeowners.

Its importance significantly increased when Witteman *et al.* in the 1995 [3] demonstrated that dust samples contain significantly high silverfish levels, even in houses where the inhabitants were unaware of its presence, and reported that silverfish is also common in Dutch indoor environments.

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Although the assay developed by Wittenman *et al.* [3] provided an important tool for establishing the presence of silverfish material in various places, silverfish allergens have not had further attention for several years. Moreover no silverfish extract for diagnosis of allergic disease is commercially available.

This article will review the latest findings about silverfish as an allergen source and deals with the issue of the preparation and characterization of an allergenic extract from silverfish insect.

Moreover, we describe the identification of the allergenic molecules, investigate the cross-reactivity among allergenic molecules from inhalant and edible invertebrates and finally the cloning and characterization of silverfish tropomyosin, the major allergenic component.

PREPARATION OF A SILVERFISH EXTRACT

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, which will be also a useful tool as a reference preparation for assessing allergen exposure in different environments.

Several different extraction methods to obtain insect allergenic extracts have been described in the literature [4-8], confirming that extraction conditions can affect the overall quality of allergenic proteins [9]. In the early 1930s insect extracts were prepared essentially according to the method of Coca and Milford [10] and an aqueous extract made up of equal parts from different insects was regarded as a useful tool suitable for *in vivo* tests such as skin, intradermal and eye test. Although since 1950 it was presumed that insect allergens could be species specific [11], and "ad hoc" extraction procedure could be needed, similar extraction procedures were employed to different insects: firstly defatting and then extracting with isotonic buffered aqueous solution. The final extract obtained was usually concentrated. Such extracts, in addition to being water soluble, were insoluble in alcohol, non dialyzable, thermostable and did not contain significant non-specific irritants. However many Authors gave few details on their extraction methods [6] and no studies have been done on the stability of these extracts, considering the possibility that certain proteolytic enzymes may be carried through during the extracting process. Therefore, during last decades, several Authors described and compared different methods of preparation of insect allergens. Usually, frozen whole insects were grounded and defatted with ether. The dried powder was extracted in an aqueous buffer and dialyzed, filtered and then lyophilized. The stable extracts obtained could be stored under desiccation until used for an indefinite period of time [9].

Similar extraction methods were carried out to obtain extracts from two different species of silverfish (*Ctenolepisma longicaudata* and *Lepisma saccharina*) [5, 7, 12]. Although several steps were added in extraction procedure, an in-depth evaluation of optimal extraction conditions and the characterization of the allergenic extract have never dealt with.

Recently we have reported an optimal extraction protocol established after evaluating different strategies [4]. Preliminary extraction experiments, carried out in an aqueous buffer at about pH 7, suggested that the resulting silverfish extracts could lack important allergenic components when tested for IgE reactivity after immunoblotting. Thus, a specific procedure was developed leading to two fractions, one obtained from an aqueous extraction at a pH close to neutrality (Sup) and the other from the insoluble material resuspended at a basic pH (Ppt).

CHARACTERIZATION OF A SILVERFISH EXTRACT

The biochemical characterization of an extract obtained from an allergenic source is the first step to identify allergenic components and to evaluate their allergenic importance. For a long time, few efforts were made in this field and more attention has been given to compare IgE reactivity to different insect species [13].

The above described extract from silverfish has been characterized by means of biochemical and immunochemical assays in order to investigate the validity of the extraction procedure, to identify the various components and to evaluate their allergenic importance. The two fractions showed a complex electrophoretic pattern and did not substantially differ in terms of protein content. 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 different protein patterns in a broad MW range from 5 to over 175 kDa. The presence of some high MW bands and of a 14 kDa component was restricted to the Ppt fraction only. Common components were detected in both fractions but with differing intensities.

Both Sup and Ppt have been also characterized by lectin blotting analysis on SDS-PAGE-separated fractions. The results suggest that most of the fractions' components contain glycosidic residues, mainly mannose and N-acetylglucosamine.

Different prevalence data were obtained when Sup fraction and Ppt fraction were used in ELISA screening with sera from two groups of subjects, one consisting of 202 randomly selected subjects and the other of 50 insect-allergic patients. 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. 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 [18], 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. In conclusion, silverfish is an important source of aeroallergens, and it should be included in the skin test bat-

tery for screening the causative allergens in patients with asthma.

IDENTIFICATION OF ALLERGIC COMPONENTS IN A SILVERFISH EXTRACT

The data reported in literature suggested that silverfish and other insect extracts, prepared by identical procedures, showed different IgE-binding patterns in immunoblotting [2, 13]. For example, the immunoblotting of *Ctenolepisma longicauda* extract developed with human specific IgE showed a lower number of components when compared with other insect extracts. The most reactive components in almost all the insect extracts analyzed, had MW ranging from 30 kDa to 37 kDa.

The IgE reactivity to the two fractions of our in-house silverfish extract, was investigated employing sera from patients allergic to arthropods, diagnosed on the basis of clinical history, skin prick test (SPT) and RAST reactivity, since a diagnostic preparation of *Lepisma saccharina* was not available [4].

First of all, the role of carbohydrate moieties in the binding of patients' IgE was further investigated by ELISA before and after periodate treatment. The results obtained 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.

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 kDa, though many other components are frequently recognized by sera, thus indicating a spread 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 kDa), 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 MW of 31–35 kDa present only in the Ppt fraction. Such component has been identified as silverfish tropomyosin [4].

Tropomyosin has been recognized as a cross-reactive inhalant/food allergen, and its pivotal role in cross-reactivity between foods and aeroallergens of animal origin has been described [14–15].

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 sources from invertebrates.

Another group of components showing MW close to 100 kDa is recognized with high frequency in the Ppt fraction and data reported in literature suggest that this component could be silverfish paramyosin [4].

Taken together, our findings indicate that the classic aqueous extraction procedure is not completely satisfactory, since important allergenic components are lost at pH close to neutrality [4].

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 a significant 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 other allergen sources from invertebrates such as cockroaches and mites.

Subsequently, we have analysed the two fractions of the silverfish extract in immunoblotting developed with monoclonal and polyclonal antibodies raised against mite and shrimp tropomyosin, respectively [16]. The use of these specific antibodies allowed us to confirm the results obtained with IgE of allergic patients and to identify for certain the presence of tropomyosin in a new allergenic source present in indoor environment that could give a possible contribution to increase tropomyosin indoor levels. This is in line with that previously evidenced by other Authors reporting that some dust samples have too high concentration of tropomyosin in function of the actual mite presence, indicating the contribution of additional tropomyosin sources other than mites [3].

ALLERGENIC CROSS-REACTIVITY

A general cross-reactivity within household insects (panallergy) and with other arthropods, especially mites, has been widely described since the earliest studies on inhalant insect allergy and it is difficult to determine whether a patient has multiple sensitivities because of multiple insect exposure or widespread cross-reactivity [14–15]. However, the last of the two explanations seems more likely especially since strong positive reactions were found to a wide spectrum of insects which are unlikely to have ever been encountered by the subjects studied. This broad cross-reactivity, and the consequent polysensitization of patients, hampers the identification of a particular insect as source of sensitization in a natural exposure. Moreover, several studies have shown that high reactivity against insects is frequently accompanied by high reactivity against mites [1]. Inhibition experiments obtained with whole insect extracts indicated cross-reactivity between silverfish, cockroach or chironomid and mite [17]. However these cross-reactions among insects extend across the class Arthropoda to include mites, crustaceans, and may even extend to other invertebrates such as molluscs and nematodes [14–15]. Cross-reactivity data obtained with whole extracts have been confirmed and implemented at the single allergen level, due to the increasing availability of purified and recombinant allergenic molecules.

Tropomyosin has been proposed as the protein responsible for this "panallergy" syndrome [18] although additional allergens could also be involved in cross-reactivity among invertebrates [19-20].

MOLECULAR CLONING OF SILVERFISH TROPOMYOSIN

The availability of recombinant allergenic molecules may contribute to the standardization of allergens used for the diagnosis and immunotherapy of allergic disease. Moreover, knowledge of the complete amino acid sequence facilitates the determination of T and B cell epitopes along the allergenic molecule [21], thus suggesting new approaches for preventing and treating allergic disease. Finally, the evaluation of sequence homology provides information on IgE-binding structures shared among close and distant species and on their biological and immunological functions, contributing from a molecular point of view to the study of allergenic cross-reactivity.

Tropomyosin belongs to a family of highly conserved proteins, some of which have allergenic activity. In fact, in contrast to vertebrate tropomyosins, invertebrate tropomyosins have been demonstrated to be allergenic [14]. Molecular cloning of tropomyosin from different sources could provide useful tools to investigate their allergenicity and cross-reactivity.

To this aim, we have cloned and expressed in *E. coli* a silverfish tropomyosin, named rLep s 1. The recombinant molecule has been characterized by means of immunoblotting with specific tropomyosin antibodies and human IgE from crustacean-allergic and house insect-allergic patients [16]. Moreover, we have studied by inhibition experiments, the correspondence between the recombinant tropomyosin and its natural counterpart in the silverfish extract corresponding to the allergenic component of 31–35 kDa recognized by tropomyosin specific antibodies and human specific IgE in the insoluble fraction of silverfish extract.

The nucleotide sequence shows 69% of homology with tropomyosin from *Homarus americanus*, and the deduced amino acid sequence shows the highest degree of homology with tropomyosin from *Drosophila melanogaster*. Moreover, a significant homology with the IgE-binding epitopes E2, E3, E4, E6 described on *Penaeus aztecus* tropomyosin by Reese *et al.* [22] has been found in the corresponding regions identified in the Lep s 1 sequence.

These significant homologies found within regions identified as critical for IgE-binding could contribute to the wide cross-reactivity among tropomyosins from different sources.

rLep s 1 is able to inhibit the IgE binding to the insoluble fraction of silverfish extract suggesting that the IgE epitopes are shared by recombinant and natural tropomyosin. A strong IgE cross-reactivity among allergenic sources from inhalant and edible invertebrates, due to tropomyosin molecule, has been extensively investigated also at level of IgE epitopes [23].

To investigate whether silverfish tropomyosin shared IgE epitopes with tropomyosins from other sources, ELISA inhibitions were performed. Tropomyosins from three arthropod sources were used as inhibitors and three human

sources were used as inhibitors and three human sera from patients allergic to arthropods were selected as IgE sources. The highest inhibiting capacity is displayed by natural shrimp tropomyosin. This result could be due to the fact that the purified preparation contains a wide range of isoforms. However, significant inhibition of IgE binding to rLep s 1 was also obtained with rPer a 7 and rDer p 10, suggesting that IgE cross-reactive epitopes shared by tropomyosins from different sources were represented on the rLep s 1 molecule [16].

This widespread cross-reactivity may result in clinical consequences. In fact, several studies indicate that the exposure and sensitization to a particular food allergen may ultimately lead to sensitization to certain aeroallergens [18] and *vice versa* [24-25]. Several Authors have attempted to identify the primary source and consequently the initial route of sensitization (ingestion or inhalation) by means of inhibition experiments. Recently, an interesting study has indirectly suggested that sensitization to shrimp tropomyosin could occur by inhalation route, on the basis of the observation of a group of Orthodox Jews who strictly observe kosher dietary laws that prohibit eating shellfish, so excluding the exposure by ingestion route [26]. It was also suggested that classic immunotherapy for house dust mite allergy may lead to sensitization to cross-reacting seafood tropomyosin [24, 27], adding a possible third sensitization route (the injective one) involved in tropomyosin sensitization.

We have evaluated the ability of rLep s 1 to trigger histamine release from basophils passively sensitized with IgE from a representative insect allergic patient. The results obtained demonstrated that rLep s 1 was able to induce histamine release at a level comparable with that obtained with the native tropomyosin, confirming its full allergen activity [16].

In conclusion, tropomyosin represents the first allergen identified in silverfish extract, or rather in its insoluble fraction. Recombinant silverfish tropomyosin, rLep s 1, can be regarded as an allergenic molecule cross-reactive with tropomyosins from inhalant and food sources. Moreover, since tropomyosin molecules can elicit hypersensitivity reactions after sensitization through three different routes (inhalation, ingestion and parenteral administration), rLep s 1 could provide a useful contribution to evaluate the role that the sensitization route plays in the development of allergy disease.

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