

Original article

Isolation and characterization of Tha p 1, a major allergen from the pine processionary caterpillar *Thaumetopoea pityocampa*

Background: Pine processionary caterpillars induce dermatitis by a toxic–irritative mechanism. The existence of true allergic reactions to allergens from these caterpillars has been recently demonstrated by positive immediate skin prick tests and specific IgE determination by immunoblotting using crude larval extracts. The aim of this work was to purify allergens from the crude larval extract in order to characterize IgE-binding proteins from these caterpillars.

Methods: Allergens were separated by ethanol gradient fractionation and reversed phase HPLC. The N-terminal amino acid sequence of a selected allergen was obtained after SDS-PAGE and transfer. The clinical relevance of this allergen was measured using sera from patients allergic to caterpillar.

Results: An allergen with a molecular weight close to 15 kDa was purified. It was recognized by 9 out of 11 allergic patients (82%). Its N-terminal amino acid sequence had no homologies to any other protein already described in data bases. For this reason, no information about its biological function could be obtained.

Conclusions: This 15-kDa IgE-binding protein is a major caterpillar allergen and shows no homologies to other insect allergens already described.

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The fact that many caterpillars are harmful to humans and animals is well known. They have even been considered as an occupational health problem (1, 2). Some of them are able to induce systemic toxic symptoms (3, 4), but in most cases the exposed patients present dermatitis (5–8), contact urticaria (9, 10) or ocular lesions (11, 12). Poisonous hairs of chitinous spines have been thought to be responsible for the production of these local lesions by a toxic and irritant mechanism (13, 14). Thaumetopoein, a protein with histamine-releasing properties has been described as an important inducer of skin lesions. This protein has a direct non-IgE mediated mechanism of action (13). A similar protein showing immunological cross-reactivity has been found in the urticating hairs of the oak processionary caterpillar (*Thaumetopoea processionea*), which explains the urticating properties of this caterpillar (14).

However, the fact that some subjects present violent reactions, such as anaphylaxis, after contact with pine caterpillars led to the discovery of an IgE-mediated mechanism. The presence of specific IgE directed to a 18 000 Da allergen, as well as to a protein with a molecular weight over 45 000 was described (15). A

severe anaphylactic shock was also reported after contact with pine caterpillars (16) as well as with oak processionary caterpillars (17). The clinical description of allergy to the pine processionary caterpillar and IgE-immunoblot patterns of several sensitized patients have been recently reported by us (18).

The aim of the present manuscript was to characterize allergens of a pine processionary caterpillar extract in order to find out their clinical relevance and obtain information about their biological role.

Material and methods

Sera

Sera from patients showing clinical symptoms after contact with processionary caterpillars and positive skin prick tests were used for the clinical relevance assays. During the purification procedures a serum pool was used. Sera from atopic patients without any symptom related to caterpillar exposure were used as negative controls.

Crude extract

The crude extract of whole caterpillars in the last larval stage (L5) was prepared by maceration in saline (1/10 w/v) and agitation for 24 h. After centrifugation for 15 min at 4500 g, supernatants were collected and stored at –24°C until use.

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Ethanol fractionation

Ten ml of the crude extract were mixed with different amounts of absolute ethanol and left for 30 min at room temperature. Thereafter, the mixtures were centrifuged at 4000 *g* for 15 min, and the pellets were discarded. The supernatants were mixed again with absolute ethanol to obtain 90% ethanol concentration. After 30 min, the mixtures were centrifuged and the pellets were resuspended in 2 ml of PBS for further purification.

Reversed-phase HPLC

The pre-purified material was loaded on a 7.8 × 250 mm Nucleosil C-4 column (Vydac, Hesperia, CA) and eluted by using a linear 5–70% acetonitrile gradient in 0.1% trifluoroacetic acid for 120 min at a flow rate of 1 ml/min. Elution was monitored at 220 nm, and fractions were collected and analysed by means of SDS-PAGE and immunoblotting.

Peak 29, showing IgE-binding properties and a molecular weight corresponding to the candidate allergen, was purified to determine the N-terminal amino acid sequence. The allergen was subjected to SDS-PAGE, transfer and selective removal of the band from a PVDF membrane (Sequi-Blot PVDF, Bio-Rad), according to the manufacturer's instructions.

N-terminal amino acid sequence

Automatic N-terminal sequence analyses were performed at Proteomics Laboratory (Centro Nacional de Biotecnología, CSIC) on a Procise Protein Sequencing System sequenator (model 494; Applied Biosystems, Foster City, CA). The sequence obtained was compared with the protein sequences in the BLAST-2 and SWISS-PROT data bases.

Specific IgE determination by means of immunoblotting

The IgE immunoblotting was used for two purposes: during the allergen purification procedures, and to evaluate the clinical relevance of the isolated allergen.

Allergen purification and characterization. The different ethanol fractions or column peaks were placed in individual wells of 16% acrylamide minigels and subjected to electrophoresis (150 V) in standard SDS-PAGE conditions. After electrophoresis, immunoblotting was performed as described in previous work (19).

Specific IgE determination in the different human samples. For specific IgE determination in the different samples, 0.1 ml of the crude extract or the purified protein was mixed with 0.1 ml of sample buffer and loaded in a minigel prepared without lanes. In order to evaluate the clinical relevance of a purified allergen, a Mini-Protean II Multiscreen (Bio-Rad) was used to study different sera from patients as described in previous work (19).

Results

The use of a crude larval extract proved to be unreliable for *in vitro* assays, because it induced artifacts during electrophoresis due to the presence of aggregates. Therefore, the solubility of the allergens in ethanol was tested. The lowest ethanol concentration assayed (28% v/v) induced the precipitation of a large amount of material leading to a better resolution of the proteins that

remained in solution. As shown in Fig. 1, the allergens appeared in the supernatant until ethanol reached a final concentration close to 60%, where the allergen with the highest molecular weight began to precipitate. Therefore, 60–90% ethanol fractionation was selected as the starting purification procedure.

Thereafter, a reversed-phase HPLC was carried out and 53 different peaks were obtained. All these peaks were lyophilized and studied by SDS-PAGE and Coomassie blue staining. Only those having molecular weights close to the allergen were assayed by immunoblotting. Figure 2 shows that one of these peaks was strongly stained by Coomassie blue, showing the presence of a contaminating protein of higher molecular weight. The human serum pool detected only the allergen and not the contaminant band. This 15-kDa allergen was not influenced by the presence of a reducing agent in the sample buffer, demonstrating that the protein had only a single chain (Fig. 3).

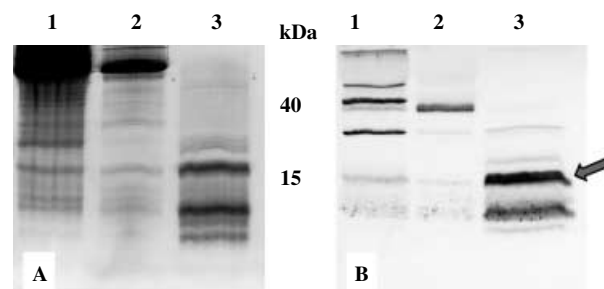


Figure 1. Ethanol fractionation (60–90%) of an 80–90% ethanol fractionated larval extract. (A) SDS-PAGE and Coomassie staining. (B) Immunoblot. Lane 1: 80–90% fractionated larval extract. The 40-kDa allergen was mainly found in the precipitate obtained in a 60% ethanol concentration (lane 2) and lower molecular weight allergens remained in the supernatant and were precipitated after raising ethanol to a final concentration of 90% (lane 3). Arrow marks the allergen selected for isolation.

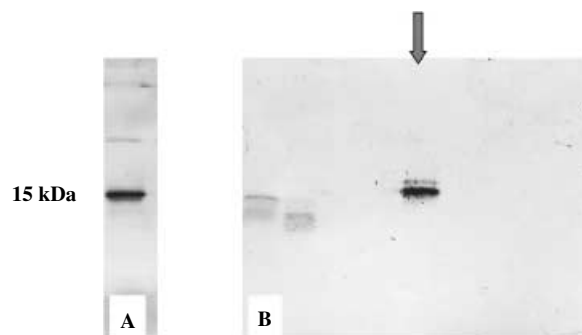


Figure 2. Reversed phase HPLC of the 60–90% ethanol fractionation. (A) SDS-PAGE and Coomassie staining. (B) Immunoblot. Only those peaks showing molecular weights corresponding to the candidate allergen were studied by blot. Arrow marks peak 29 of the HPLC fractionation selected for N-terminal amino acid sequencing.

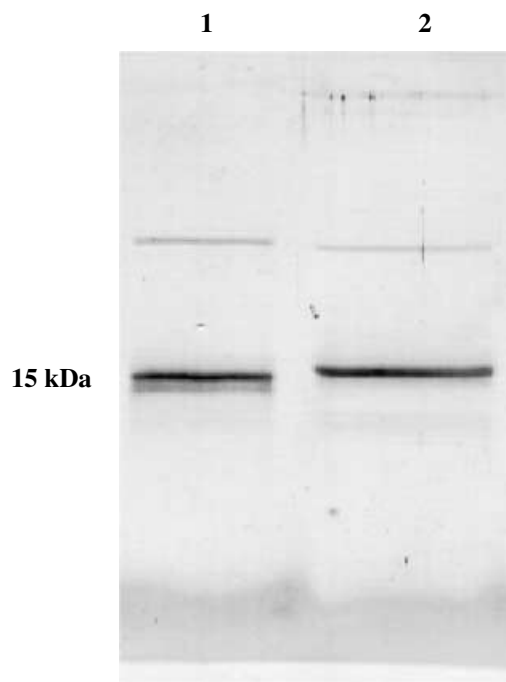


Figure 3. SDS-PAGE and Coomassie staining of peak 29 under non-reducing conditions (lane 1) and after treatment with 2-mercaptoethanol (lane 2).

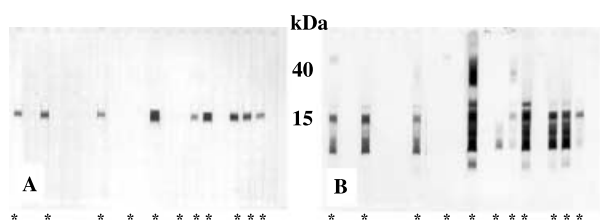


Figure 4. Clinical relevance of the isolated allergen. Immunoblotting using 11 sera from patients clinically sensitive to the caterpillar (marked with asterisk) and nine sera from atopic patients without any symptom related to caterpillar exposure, used as negative control. (A) Purified protein. (B) Crude extract.

The clinical relevance of the isolated allergen was tested using 11 sera from patients clinically sensitive to the caterpillar and nine sera as negative controls. As shown in Fig. 4, nine sera (80%) recognized this allergen. This protein was therefore considered as a major caterpillar allergen by the Allergen Nomenclature Sub-Committee of the IUIS and named Tha p 1. The terminal amino acid sequence obtained was GETYSDKYDTIDVNEVLQ (accession number A59396, PIR). No homologies to other known proteins were found in the BLAST-2 and SWISS-PROT data bases.

Finally, the behaviour of a patient sensitized to other species of caterpillar when analysed with a crude pine processionary extract and Tha p 1 was studied. Figure 5 shows the IgE-immunoblot pattern obtained using the

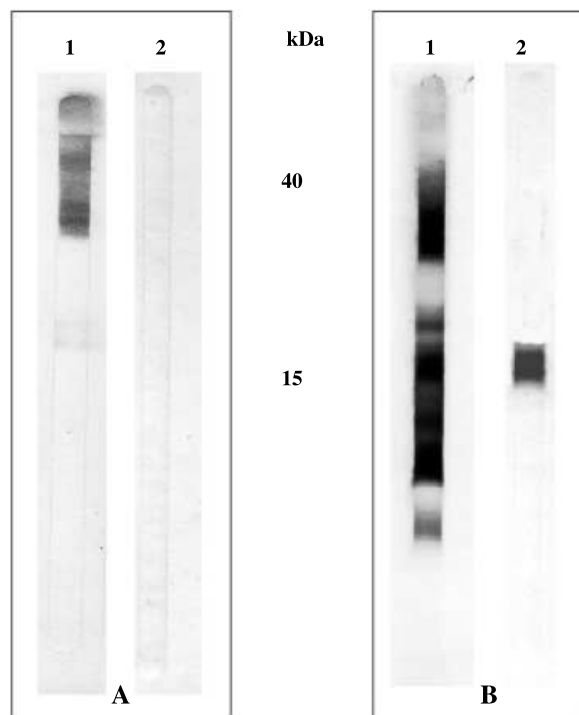


Figure 5. (A) IgE-immunoblot of a patient with immediate urticaria after contact with elm caterpillars. Although this patient detects some high molecular weight allergens in the crude pine caterpillar extract (lane 1), no detection of Tha p 1 is found (lane 2). A patient with immediate urticaria after contact with pine processionary caterpillars is shown in part B. Lane 1: crude pine caterpillar extract. Lane 2: Tha p 1.

serum of a patient who presented immediate urticaria after contact with an elm caterpillar (*Euproctis chrysorrhoea*). As shown, this patient did not recognize the 15-kDa allergen of the pine caterpillar.

Discussion

Pine processionary caterpillars are a medical problem in Mediterranean countries, Central Europe, the Middle East and North Africa. In spite of the toxic and irritative effects on skin, an IgE-mediated mechanism has been previously demonstrated as inducer of clinical symptoms in a high proportion of affected patients (18). Therefore, an allergic hypersensitivity (20) must be always suspected when a patient presents symptoms after contact with pine trees. In our previous work, we observed that, as frequently found with immunoblotting assays of other allergens, the patients showed different detection patterns. However, some allergens were more frequently detected than others. The frequency of recognition of the different IgE-binding proteins present in a crude extract has led to the description of major and minor allergens. This nomenclature is considered as unsatisfactory by some authors, because this definition does not reflect the

contribution of the allergen to the overall reactivity of the crude extract (21). In our case, Tha p 1 was not only frequently recognized by the sera, but was also strongly detected, and this fact suggested a high clinical relevance of this allergen.

The first report of allergens from the pine processionary caterpillar described a protein denominated thaumetopoein (13). Thaumetopoein was first described as a non-IgE mediated mast cell degranulator with a molecular weight of 28 kDa, composed of two subunits of 13 and 15 kDa (13). Subsequently, the same authors reported the presence of IgE directed to allergens from a pine processionary extract and described thaumetopoein as a 18-kDa protein (15). We estimated the molecular weight of Tha p 1 to be around 15 kDa, with only a single chain as seen by 2-mercaptoethanol treatment. The amino acid sequence of thaumetopoein is unfortunately not available for comparing the sequences.

The same cited authors found another allergen with a molecular weight over 45 kDa. Interestingly, an allergen

with a similar molecular weight was detected by the serum of a patient who had immediate urticaria after contact with an elm caterpillar, but there was no recognition of Tha p 1. Testing more patients with symptoms produced by other caterpillars would allow us to know if a crude pine caterpillar extract could serve as a general diagnostic tool, whilst purified Tha p 1 could be a more specific reagent for pine processionary caterpillar sensitivity. Alternatively, the same information could be obtained by immunoblotting using the crude total extract, but patients sensitized to other caterpillars were not available at the time of the study.

No information about the biological function of Tha p 1 could be derived from the N-terminal amino acid sequence, due to the fact that no homologies to other described proteins were found. This fact implies that no relationship to other previously described insect allergens exists, and increases the allergological interest of this protein.

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