

Evaluation of silkmoth (*Bombyx mori*) allergens in sericulture grainage industries as major pollutants

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Allergens from scales and cuticles of abdomen and wing of silk moth, *Bombyx mori*, causatives of allergic rhinitis have been subjected to solubilization with SDS (2%), urea (8M) and guanidine hydrochloride (6M). Allergens (70%) were extracted and all the solvent extracts contained an approx similar complement of allergens. Allergen composition of abdomen and wing scales differ from one another and both revealed 30-40 polypeptides, on SDS-PAGE separation, of which about 8 in each case make up the major proportion. Major allergens had molecular weights of 14000 to 65000. Proteins extracted from both scales represent a true reflection of the abdominal and wing cuticles and also minor differences were found between the proteins of segment and intersegment cuticle. The isolated allergens were used for immunological analysis. However, some structural components were found common to some of the allergens because of their good antibody cross reactivity.

Keywords: Silkmoth, Insect allergen, Allergic rhinitis, Cross reactivity, Immune specificity

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Introduction

The workers of silk industry throughout the world are often getting allergies due to the presence of humidial environment, which favours the growth of allergy causing organisms like *Aspergillus fumigatus* and liberation of allergy causatives during the developmental stages of silkmoth. Insect allergens have been identified as one of the major cause of asthma and allergic rhinitis¹⁻³. The insect cuticles and scales of both abdomen and wing serve functions like protection, support for musculature and specialised appendages. In the sense cuticles and scales of the insect and their growth depends on the precise timing of the shedding of the old scales and the synthesis of a new ones. The cuticles and scales of insect are a mixture of chitin and other proteins making up to 25 per cent of weight. The entire cuticle and scales are the product of a single layer of epidermis. The cuticle of the insect produces different types of the scales depending on the stage in the life of insect. Morphologically for Lepidopterans, the soft translucent white cuticle of the larva is different from the brown, brittle pupal cuticle and both bear no resemblance to the adult moth cuticle with its scales. It has been hypothesized for a long time that insect cells that produce different varieties of cuticles associated with scales under hormonal influence should have different genetic programmes which are brought into expression at different times⁴. The morphological differences between the cuticles, scales of abdomen and wing might lie in the types of cross links made between the proteins or proteins and chitin.

Analysis of cuticles from different developmental stages of *Tenebrio molitor* and *Drosophila melanogaster* demonstrate a unique set of proteins, which are specific for each stage^{5,6}. Presently, these proteins of adult moth were posing very severe threat to sericulture industrial grainage workers in causing allergic rhinitis, which is one of the most common disease in both developing and developed countries. Sericins from silkworm cocoon, moth and butterfly share a common allergenicity and silk allergy is caused not only by occupational but also by environmental exposure to the insects⁷. Silkworm products like fibroin manifests inflammatory response associated with Type-I allergy⁸. Cuticular and scale associated proteins are supporting morphological developments in the adult life of the silkworm⁹. Identification of Val, Ala and Pro (VAP-peptide) is essential for the completion of adult cuticle construction. Thus the expression of this gene provides a new model to analyze molecular mechanisms of terminal adult differentiation¹⁰. In view of associative allergy causing products from silkworm, authors have characterized the protein allergens of surface appendages like abdominal and wing cuticles and scales. The present paper deals with cuticles, scales of abdomen and wing allergens as causative

agents of allergic rhinitis in sericulture grainage industrial workers.

Materials and Methods

Insects and Animals

Bombyx mori pupae and adult moths were obtained from sericulture grainage industries and stored at 4°C for at least 8 weeks before use. Pathogen free albino mice (20-25g, 4-6 week old) were procured from National Institute of Nutrition (NIN), Hyderabad. The animals were maintained at 23-26°C, with 12 h dark and light; water and food were given following the guidelines of laboratory animal user manual of NIN.

Chemicals

All electrophoresis reagents were from Sisco Research Lab, Mumbai; Sigma, USA. Ultrapure urea, SDS and guanidine hydrochloride were from Genie, Bangalore, and Immunochemicals and reagents from Sanjay Gandhi Post Graduate Institute of Medical Sciences Lucknow.

Extraction of Allergens from Abdominal, Wing Cuticles and Scales

Animals were dissected in dissection buffer (50 mM Tris-HCl; pH 7.5; 5 mM MgCl₂; 1 mM EDTA; 0.25 M sucrose and phenyl thiourea for saturation to inhibit endogenous tyrosinase). The abdomen was separated from rest of the body. Elimination of abdomen-attached fat body, muscle was carried out in the dissection buffer and the cuticular pieces transferred to moist filter paper and frozen.

The soft wings of silkmoth were separated by gentle agitation in buffer and scraping the surface to dissociate small wing scales. Scales collected from large number of wings were concentrated in a centrifuge at 3000 rpm for 10 min at room temperature. Wings of silk moths were homogenized in PBS and centrifuged at 15000 rpm for 30 min and this extract was used as crude allergen.

Abdominal cuticles were stretched and laterally cut at each segment - intersegment border and were lyophilized. The lyophilized and frozen cuticles were cut into small pieces and then ground to powder, 5-10 mg of which was mixed with extracting solution [50 mM Tris HCl (pH 7.5), 1 mM EDTA, 1 mM β-mercaptoethanol and 2% (w/v) SDS] in the ratio of 1:5 (dry weight). The suspension was allowed to stand for 1 h at 45°C and then placed for 3 min in a boiling water bath. The mixture was cooled, centrifuged and supernatant was collected and dialyzed overnight against 0.2% SDS in 10 mM Tris HCl (pH 7.5), 1 mM EDTA and 1 mM β-mercaptoethanol. For comparative studies, abdominal, wing scales and cuticular powders were extracted with 8M urea and 6M guanidine hydrochloride.

Immunological Procedures

Mice were immunized with adult abdominal and wing scales extracts. Immunizational protocol included injection of 75 µg of protein with complete Freund's adjuvant (Genie, Bangalore) two times at 10-d interval and twice more in 0.9% (w/v) saline. Blood was collected from immunized mice after 20 - 25 days. The abdominal and wing scale proteins were separated by SDS-PAGE on gels (12.5%) and the polypeptides transferred by electro blotting to nitrocellulose sheets. Reactivity of the separated polypeptides with the antibodies was tested¹¹ by using peroxidase coupled goat anti-mouse rabbit IgG.

Protein Determination

Small amounts of cuticles, scales of abdomen and wing extracts were precipitated with 10% (w/v) TCA and washed several times with 5% (w/v) TCA, ethanol-diethylether 1:1 (v/v) and diethyl ether. The washed pellets were dissolved in 1 M NaOH at 37°C for 1 h. A modified procedure¹² was employed on aliquots of the solubilized material and γ globulin (Bio Rad) was used as a standard.

Electrophoresis

Separation of cuticles, scales of abdomen and wing proteins were routinely carried out by SDS-PAGE analysis following the procedure of Laemmli¹³ with separating gel (12.5%) and stacking gel (3%). For the isolation of individual polypeptides, 20-30 µl of cuticular and scale extracts along with sample buffer was

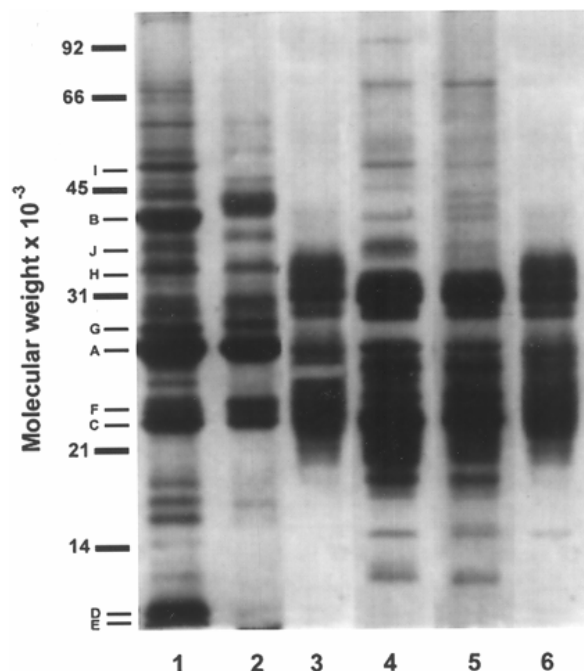


Fig. 1—SDS-PAGE analysis of proteins extracted from abdomen and wing scales with different solvents, Lane 1: Wing scales with SDS; Lane 2: Wing scales with Urea; Lane 3: Wing scales with guanidine hydrochloride; Lane 4: Abdominal scales with SDS; Lane 5: Abdominal scales with Urea; Lane 6: Abdominal scales with guanidine hydrochloride

layered on the top of the stacking gel (number slots) and run as usual. At the end of the run, longitudinal pieces were cut from both the ends and the central portion kept frozen. The cut pieces were stained with coomassie blue, destained and aligned. The gels were stained by the Silverstain technique^{14,15}.

Results and Discussion

Extraction of Abdominal and Wing Scale Proteins

The high salt extraction, which has been employed previously (1.0 M Tris HCl, pH 7.5, 0.5 M KCl)¹⁶, yielded only 4-5 per cent (dry weight) soluble material (Table 1). Since further analysis by electrophoresis involved denaturing gels, extraction was carried out with protein denaturing agents (SDS, urea and guanidine hydrochloride), which extract similar quantities of protein from the scales and cuticles (Table 1). The percentage was usually smaller for scales or adult abdominal cuticle, as it is difficult to grind these to a fine powder.

Comparison of the Abdominal and Wing Scales (AWS)

AWS extracts using any of the denaturants contain proteins of similar electrophoretic behaviour from the same wing scale with minor variations in the extent of extraction of some polypeptides (Fig. 1). The major exception is band B observed in scales extracted with SDS but not with urea, which is not as good an extractant as SDS since several small protein allergens and large ones are not brought into solution from scales (lane 2). This may be probably due to the physical nature of the abdominal scales, since wing scales yield the same pattern whether the extractant is urea or SDS (lane 4 & 5). The guanidine hydrochloride extract of SDS-PAGE pattern reveals diffuse nature of some bands. AWSs contain a large number of protein allergens (35 in wing scales, 30 in abdominal cuticle) with molecular weight ranging from 10000 to more than 100000. About 10 polypeptides (marked A-J in decreasing order of intensity of staining) unique to each stage comprise the major proportion of the total extract (Fig. 1). Due to the crowding of the protein allergens in the 20000 to 30000 molecular weight range and the slight variation in their mobility from gel to gel, the average molecular weights of major bands have been calculated from the five runs to be abdominal protein allergen A to allergen J.23, 31, 33, 30, 28, 27, 19, 17, 25 and 13 and wing allergens A-J, 28, 43, 22, 12, 11, 23, 30, 34, 54 and 41.

Attempts have been made to obtain cuticular proteins from a variety of insects and compared them either by amino acid or electrophoretic analyses^{9,16-18}. The varied results were mainly due to the various solvents utilized to extract the proteins of allergens (Water, salt, urea, acetic acid, ammonia, formic acid, formamide and NaOH) and the non-uniformity of the nature of the scales and cuticle employed. The solubility of proteins depends on the bonding of the polypeptides to other cuticular components, principally chitin and the degree of sclerotization. Since analysis of the extracted scales and cuticular polypeptides are normally carried out by SDS-PAGE or isoelectric focusing in urea, it was decided to utilize these denaturants directly for extraction. It has also been possible to precisely time the isolation of wing cuticle as it is easily seen when sclerotization begins at the edges. Isolation of scales before any melanization or hardening is easy by isolating wings from developing adults at the appropriate stage. To isolate scales and cuticles several extraction procedures were employed and a comparison of the total yield and composition of the polypeptides (Table 1) with different solvents were used (Fig. 1). Quantitation of the amount of chitin present in *Antheria polyphemus* cuticle is not available. Andersen¹⁶ surmised the dry weight of chitin (20-50%) in the procuticle of insects. Assuming the amount of chitin in *A. polyphenus* cuticles to be 35 per cent, this procedure results in a recovery of 45-50 per cent of protein present.

Table 1 — Solubilization of abdomen, wing scales and cuticular polypeptides

		Percent dry weight of scales and cuticles extracted as protein			
		High salt	SDS (2%)	Urea (8M)	Guanidine hydrochloride (6M)
Abdominal	Scales	3.2 (0.6)	26.8 (4.2)	26.5 (4.0)	28.8 (5.0)
	Cuticles	3.0 (0.8)	27.0 (4.0)	25.2 (1.4)	26.4 (4.2)
Wing	Scales	4.0 (0.6)	30.3 (2.6)	30.3 (4.3)	31.9 (3.6)
	Cuticles	3.5 (0.4)	31.1 (3.9)	29.4 (1.5)	31.5 (2.5)

Each (5-10) mg of cuticles, scales of abdomen and wing was extracted separately by standard deviation and mean methods. Protein analyzed from soluble material after precipitation with TCA and solubilization in 1M NaOH (average 3 determinations).

The numbers in parentheses are $\pm$ standard error of the mean.

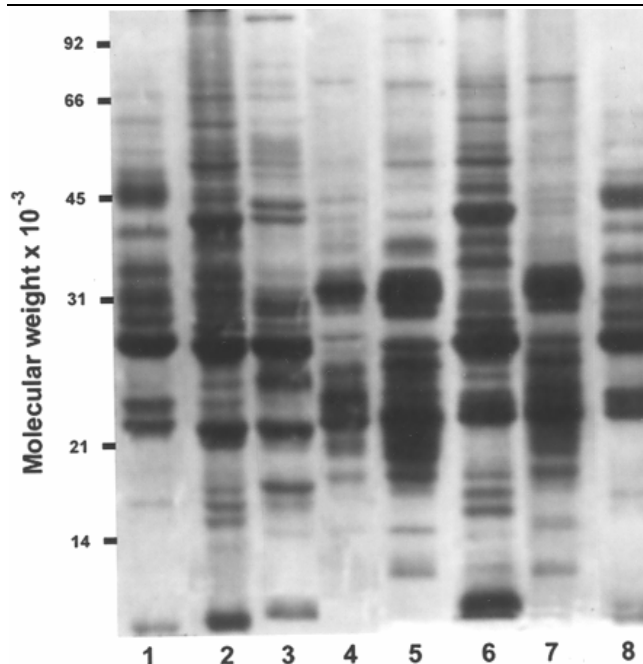


Fig. 2— Comparison of abdomen and wing cuticular extracts with scale extracts, Lane 1: Abdominal cuticle with SDS; Lane 2: Wing cuticle with SDS; Lane 3: Abdominal cuticle with urea; Lane 4: Wing cuticle with urea; Lane 5: Abdomen scales with SDS; Lane 6: Wing scales with SDS; Lane 7: Abdomen scales with Urea; Lane 8: Wing scales with Urea

Comparison of Abdominal and Wing Cuticles (AWCs)

While the wing epidermis functions only during the pupal and adult stages with no equivalent at the larval stage, the abdominal epidermis persists throughout development and secretes different cuticles at the larval, pupal and adult stages. Cuticles and scales derived from wing and abdomen, demonstrate similar protein allergens characteristic of the particular stage irrespective of the extractant (Fig. 2). Urea seems to be less efficient than SDS in extracting all the protein allergens.

The abdominal cuticle is dark, soft and full of scales. However, intersegmental region remains thin and transparent. Despite these major morphological differences, the polypeptides comprising the segments and intersegments are similar to the cuticle from which they were derived (Fig. 3). The ventral abdominal cuticle was used for

total extracts while the segments and intersegments were derived from the dorsal side of the same animals. The pattern obtained from the total adult abdomen (AAb) shows most of the bands except B (Fig. 1). Again, the patterns seen with segments (AS) and intersegments (AIS) are similar to moth abdomen. Minor differences of certain bands, seen in AS and AIS, might be due to the limitation of the amount of the protein

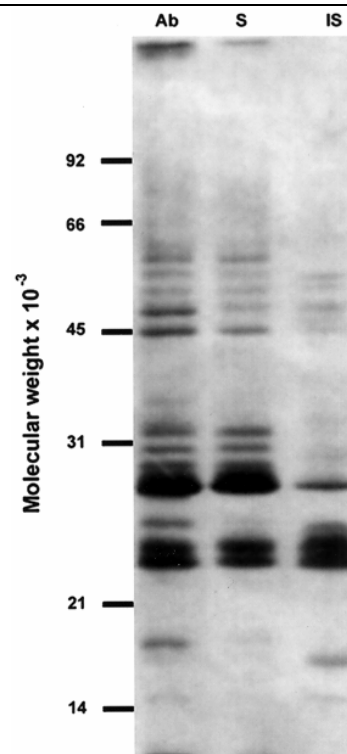


Fig. 3— Comparison of proteins in the cuticular extracts from total abdomen with those from segments and inter segments. Ab - Abdomen, S - Segment, IS - Intersegment

that was available as well as inability to grind to the powder prior to the extraction.

More similarities than differences were observed in the electrophoretic pattern of abdomen and wing scales and cuticular polypeptides from different stages of development¹⁸⁻²⁰. However, Robersts and Willis⁶, while showing that several bands were similar in extracts from larval and pupal cuticles of the mealworm *Tenebrio molitor*, also showed that these two patterns of proteins were more similar to each other than to those of proteins from adult cuticles. Therefore, it was possible to recognise each stage from differences in the gel patterns and relative intensities. The results from the analysis of cuticles, scales of AWCs from the silkmoth *B. mori* confirm and agrees some of these conclusions. One major difference between *B. mori* cuticles and scales with other studies seems to be in the size of the cuticular proteins^{5, 10, 11, 21}. The cuticular

peptides from *Tenebrio molitor*¹⁰ and *Sarcophaga bullata*²¹ seem to have molecular weights below 25000. In contrast, all major polypeptides in the silkmoth *B. mori* cuticles and scales have molecular weights above 20000. When the different scale and cuticular preparations were run simultaneously, one would find several bands that migrate with similar molecular weights. This does not, however, mean that they are similar polypeptides, which have been normally assumed.

The adult cuticle consists of smooth cuticle and the characteristic scales of the moths and butterflies. Scales, although structurally distinct from the smooth cuticle, are also the results of epidermal cell differentiation and cuticle secretion, since both arise from different cells, but have a common origin. It was of interest to determine whether the composition of two differ radically or are they similar. Although, the present polypeptide analysis of scales and abdominal cuticles from the silkmoth confirmed this (Fig. 3). This analysis leads to a further conclusion that the cuticle as a product of the epidermis, no matter its location in insect body, reflects the various stages of the entire organism (Fig. 2). Evidence to support this conclusion is, of course, a comparison of abdominal and wing scales, which form the major portion of AWCs and also the similarity in the pattern, obtained with segments and inter segments (Fig. 3). There do exist minor differences in the polypeptide pattern for example the band at 45 kDa which is observed in extracts of scales abdomen and segments being very low are absent in intersegments might be specific for scales.

Immunological Analysis

Ascites fluid obtained from mice immunised with the abdominal and wing scale extracts showed good cross reactivity against both the extracts in the form of precipitin lines by Ouchterlony technique. Due to the complexity of the polypeptides, cross reactivity was measured by blotting technique, which helps in identifying specific polypeptides that do not cross react. The coomassie stained lanes shown abdominal and wing cuticular proteins (Fig. 4). Homologous antibodies show cross reactivity with almost all the bands observed in present study. The antibodies raised against wing scale proteins react with several abdominal polypeptides especially in the 14000 - 31000 molecular weight range. Similarly, the antibodies against abdominal proteins react with several wing polypeptides. In both instances, the protein band around the 30000 molecular weight position is the more reactive. Control ascitic fluid did not show any reactivity.

The common features are observed by the antibodies, as reflected by good cross reactivity of abdominal scale proteins with antibodies to wing scales and vice versa. The cross reactivity is neither as intense nor does it cover the entire complement of proteins in either case (Fig. 4). It is necessary to compare the polypeptides with similar molecular weights that show cross reactivity by other techniques to establish the relationships involved.

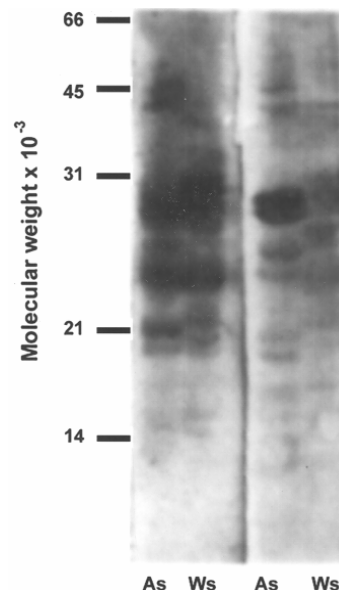


Fig. 4— Immunological reactivity of abdomen and wing scales and cuticular polypeptides with antibodies against abdominal and wing scales and cuticular extracts. Lane 1: Proteins stained with coomassie blue; Lane 2: Antibody to abdomen and wing scale proteins; AS-Abdominal Scales, WS-Wing Scales, Y-axis: Molecular weight $\times 10^{-3}$

Conclusions

Present results call attention to silkworm moth allergens of abdomen, wing scales and cuticles as causatives of allergic rhinitis in grainage industrial workers in India.

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