



How Allergen Extracts Are Made—From Source Materials to Allergen Extracts

Allergenic extracts to diagnose and treat sensitivity to insect venoms and inhaled allergens

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ABSTRACT

Objective: To review allergenic extracts used to diagnose or treat insect allergies, including how the extracts are manufactured and their measurements of potency or concentration.**Data Sources:** Peer-reviewed articles derived from searching PubMed (National Center for Biotechnology Information) about insect allergies and extract preparation. Encyclopedia of Life (<http://www.eol.org/>) and <http://allergome.org/> were also referenced for background information on insects and associated allergens.**Study Selections:** Search terms used for the PubMed searches included *insect allergens and allergies, Apidae, Vespidae, fire ants, cockroach allergies, insect allergen extract preparation, and standardization*.**Results:** Humans may be sensitized to insect allergens by inhalation or through stings. Cockroaches and moths are predominantly responsible for inhalation insect allergy and are a major indoor allergen in urban settings. Bees, fire ants, and wasps are responsible for sting allergy. In the United States, there are multiple insect allergen products commercially available that are regulated by the US Food and Drug Administration. Of those extracts, honeybee venom and insect venom proteins are standardized with measurements of potency. The remaining insect allergen extracts are nonstandardized products that do not have potency measurements.**Conclusion:** Sensitization to inhalational and stinging insect allergens is reported worldwide. Crude insect allergen extracts are used for diagnosis and specific immunotherapy. A variety of source materials are used by different manufacturers to prepare these extracts, which may result in qualitative differences that are not reflected in measurements of potency or protein concentration.

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Introduction

Insects sensitize humans by 1 of 2 routes: inhalation or stings. The most common inhalational sensitizers are German and American cockroaches. Moths, butterflies, and silk worms may also sensitize by inhalation, but the most common offenders are cockroaches, which are major allergens in urban settings. Along with house dust mites, cockroaches may initiate the allergic march toward allergic asthma.¹

Thousands of species of bees, wasps, and ants are capable of stinging when aggravated. Hymenoptera venom allergy refers to

local or systemic allergic reactions in response to insect stings belonging to the order Hymenoptera. Hymenoptera populations vary by region. In Central and Western Europe, Hymenoptera venom allergy is primarily induced by yellow jacket or honeybee stings and less frequently by hornets or bumblebees. In parts of Southern Europe, paper wasp stings are most prevalent. In the United States, honeybees, wasps, and ants may induce allergic sensitization.²

Venom allergy after an insect sting is a classic example of cross-linked receptor-bound IgE antibodies that induce mast cell degranulation. Allergic reactions to venom may be localized to the site of the sting (swelling, redness, or itching) or systemic. Stings or bites from mosquitos, flies, ticks, and midges induce specific IgE but primarily cause local allergic reactions and therefore are not considered a major public health concern in allergic disease. By contrast, venoms from Apidae (honey and bumblebees), Vespidae (wasps such as yellow jackets, paper wasps, and hornets), and Formicidae (fire ants) are responsible for more than 10% of systemic allergic reactions and approximately 40 deaths in the United States each year.^{3,4} Sting allergies may occur at any age but are less frequent in children than adults. Up to 5% of Apidae and Vespidae

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stings induce IgE-mediated hypersensitivity, and up to 32% of honeybee keepers are sensitized to honeybee venom.^{2,5} We review the insects responsible for inhalational and venom allergy in the United States and elsewhere. We also review the extracts used to diagnose or treat venom allergy, including how they are manufactured and their measurements of potency or concentration.

Inhalational Insect Allergens

Cockroach

Inhalational allergy to cockroach was first reported in 1964.⁶ There are more than 5,000 species of cockroaches that inhabit tropical forests and are not major inducers of inhalational allergy. By contrast, German and American cockroaches (*Blattella germanica* and *Periplaneta americana*, respectively) have adapted to cohabitate with humans and are the major source of perennial environmental indoor allergens in urban and low socioeconomic settings.^{7,8} The prevalence of allergic sensitization to cockroach allergens in urban homes is high. More than 65% of all inner-city children and 35% of those who have asthma are allergic to cockroaches.⁹

Cockroach excretory products contain several allergens. In addition, body secretions and egg casings dry and become a component of airborne indoor dust that remain in the environment for several months after cockroaches are removed.^{10,11} Unlike cat dander and ragweed pollen, there are no cockroach allergens that dominate the allergic response. Instead serum samples from cockroach allergic patients reveal complex patterns of reactivity to multiple proteins with no consistent patterns that typify allergic sensitization.¹⁰ There are more than 10 groups of cockroach allergens (www.allergen.org) listed in the World Health Organization/International Union of Immunological Societies allergen database, but novel allergens have been identified in cockroach feces and whole bodies.^{11–13} In addition to initiating an allergic response, proteolytic activity of some allergens may add to the severity of cockroach sensitization by disrupting the integrity of airway epithelial cells.^{14,15}

A method of manufacturing cockroach allergen extracts starts with culturing cockroaches in a closed moist container that contains the insects and feed. The whole bodies, along with their secretions and excretions, are collected and killed by freezing. The killed frozen insect bodies serve as the source material for allergen extraction. After thawing, the bodies are milled to a fine powder, defatted with acetone, and air dried. The material is then mixed with an extraction buffer, which is usually glycerol and Coca solution or phosphate-buffered saline. The extraction is usually performed at 2°C to 8°C, after which the extracted material is centrifuged to remove solid particles.^{7,16} Sometimes the bulk-extracted material is stored for few hours to days to allow precipitation of additional proteins before dispensing into vials for long-term storage. As may be inferred from its manufacturing process, cockroach extract is highly complex and may vary, depending on the chow fed to the roaches and the differences in the method of separation of roaches and their secretion and excretion products from the chow.

Cockroach allergen extracts are not standardized for potency, and therefore the finished vials are only labeled as weight/volume, which refers to the amount of source material in grams and the volume of extraction solution in milliliters. As is the case for all nonstandardized extracts, there are no controls for the composition and potency of cockroach extracts. The variability of protein concentration, fraction of allergenic proteins, and proportions of allergens differ among cockroach allergen extracts.⁷ This variability among cockroach extracts and lack of standardization may contribute to the poor efficacy of cockroach allergen immunotherapy.¹⁷ Complicating the complexity of cockroach allergen extracts is the presence of glycinin, a soybean allergen homolog, in a German cockroach fecal extract.¹¹ Rather than a component of the

roaches, glycinin is likely a contaminant from the rodent chow fed to the cockroaches. If the extracts containing glycinin are used to diagnose cockroach allergy, then patients who are soybean sensitive may be incorrectly diagnosed as being allergic to cockroaches.

Silkworm Moth

The silkworm moth (*Bombyx mori*) is completely domesticated insect that cannot survive in the wild.¹⁸ Silkworm moths are grown as sericultures for recovery of silk, and silkworm pupae are a popular traditional food in much of Southern and Eastern Asia. The moths are traditionally perceived as a source of occupational inhalant allergens in silk-producing industries. Silk and silk waste products used as filler in bed mattresses contain several IgE-binding allergens and are also responsible for contact allergies.¹⁹ As many as 40% of patients with respiratory allergy in Guangzhou, Southern China, are sensitized to silkworm moth. Almost 50% of those patients are also sensitized to 1 of 9 additional inhalant allergens, including 3 species of house dust mites (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, and *Blomia tropicalis*) and German and American cockroach.²⁰ In addition, those who are allergic to silkworm moths are often sensitive to other moths and butterflies.²¹

Compared with cockroach allergens, silkworm moth allergens are poorly defined. Arginine kinase (Bomb m 1, 42 kDa) has been identified as a major silkworm allergen and is cross-reactive to a similar cockroach allergen.^{19,22} Multiple allergens with molecular weight ranging from 14 to 70 kDa are described in the components of silkworm moth waste, which is used as a mattress filler.²³ In 2016, Jeong and colleagues²⁴ identified a heat stable 27-kDa glycoprotein as an allergen in silkworm pupae extract that was boiled for 5 minutes.²⁴

Crude allergen extracts of *Bombyx mori* are prepared from the wings covered with scales. Abdominal cuticular pieces can also be used as an allergen source. The collected material is cut into smaller pieces before maceration and defatting with ethyl ether or acetone. For allergen extraction, the powdered material is mixed with buffer solution (1:10 or 1:20 wt/vol ratio) and allowed to mix for 24 to 48 hours at 2°C to 8°C. The extract is then centrifuged and the supernatant filtered before use for skin testing.²⁵

Stinging Insect Allergens

There are 3 families of stinging insects: Apidae (honeybee, bumblebee), Vespidae (yellow jacket, yellow hornet, white-faced hornet, paper wasp) and Formicidae (fire ant, harvester ant, jack jumper ant) (Table 1).

Honeybee Venoms

Wild honeybees (*Apis mellifera*) nest in tree hollows, rotted logs, and occasionally in voids in walls of homes and other buildings. Honeybees are generally not aggressive and sting only when they sense danger to the nest or the queen. IgE-mediated anaphylaxis to honeybee venom is one of the most severe forms of Hymenoptera venom allergy. Honeybee sting reactions range from local reactions to anaphylactic shock and death. As reviewed by Bilo and Bonifazi,²⁶ the prevalence of systemic reactions in adults to Hymenoptera stings ranges from 0.5% to 3.3% in the United States and 0.3% to 7.5% in Europe. Among those who are allergic to honeybee venom, 0.3% to 42.8% of systemic allergic reactions were considered life-threatening (ie, classified as anaphylaxis).

Although honeybee venom has historically been considered well characterized, 2 research groups used proteomic methods to identify more than 80 peptides or proteins in honeybee venom, of which 4 are proteins that are potentially novel allergens.^{27,28} Among the 12 allergens that are well described, the 3 major honeybee venom allergens are phospholipase A2 (Api m 1),

Table 1
Standardized insect venom or venom protein licensed for distribution in the United States approved by the US Food and Drug Administration

Venom or venom protein		Source ^a	Units	Major allergens (kDa)
Honeybee venom		<i>Apis mellifera</i>	Total protein concentration in micrograms per vial	Api m 1 (16) Api m 2 (44) Api m 4 (3)
Yellow jacket venom		<i>Vespula</i> species	Total protein concentration in micrograms per vial	Ves 1 (33) Ves 2 (44) Ves 5 (23)
Wasp venom		<i>Polistes</i> species	Total protein concentration in micrograms per vial	Pol 1 (33) Pol 2 (44) Pol 5 (23)
Yellow hornet venom		<i>Dolichovespula arenaria</i>	Total protein concentration in micrograms per vial	Dol a 1 (35) Dol a 2 (38) Dol a 5 (23)
White-faced hornet venom		<i>Dolichovespula maculata</i>	Total protein concentration in micrograms per vial	Dol m 1 (35) Dol m 2 (44) Dol m 5 (23)

^aImages are obtained from Encyclopedia of Life (www.eol.org).

hyaluronidase (Api m 2), and melittin (Api m 4), a small-molecular-weight protein.^{29–31} Additional allergens include 2 highly similar glycoproteins that are collectively labeled Api m 11 (major royal jelly proteins 8 and 9), to which IgE binds to both protein and carbohydrate domains.^{32–34} In addition, honeybee venom includes many enzymes, including the allergens acid phosphatase and dipeptidyl peptidase (Api m 3 and Api m 5, respectively), and a protease inhibitor (Api m 6). Because these enzymes or inhibitors may affect protein integrity, honeybee venom should not be mixed with any other allergenic extract.^{33,34}

Crude honeybee venom is used in the manufacture of allergen extracts. Honeybee venom allergen extracts are used to diagnose Hymenoptera venom allergy and for specific immunotherapy. Honeybee venom is usually collected by applying an electric shock through a grid that is placed adjacent to the entrance of the hive and over a collection apparatus (a polyethylene sheet stretched over a glass plate). The hive is disturbed by tapping, poking, or pushing it near its entrance. The first few honeybees that fly out of the hive encounter the grid and are shocked by the voltage applied. The honeybees then sting the grid and eject venom into the collecting apparatus. The first honeybees that sting the grid also release an attack pheromone that induces the other bees to leave the hive and sting the grid. A hive is milked for 15 to 20 minutes, after which the voltage is switched off. Once the hive has calmed, the collection apparatus is removed, and the venom is collected from the glass plate and polyethylene sheet. After ultrafiltration, the honeybee venom is freeze-dried.³⁵ (For further information on the honeybee venom collection process see http://www.ibiblio.org/pub/academic/agriculture/entomology/beekeeping/general/venom_therapy/).

In the United States, honeybee venom allergen extracts are standardized. Each final container vial contains 100 µg of freeze-dried venom that is to be reconstituted to a volume of 1 mL for a concentration of 100 µg/mL. In addition to total protein, commercial allergen extract manufacturers are required to report the concentration of hyaluronidase and phospholipase A2 (in units per

milliliter of each), both of which are measured by an agarose diffusion assay using a freeze-dried venom provided by the US Food and Drug Administration Center for Biologics Evaluation and Research as a reference reagent.³⁶

Yellow Jacket and Paper Wasp

Yellow jacket (*Vespula* species) and paper wasp (*Polistes* species) nests are composed of a paperlike material or wood pulp and generally reside in concealed locations, such as under eaves, behind shutters, and in building frames and wall cracks. Unlike honeybees, the Vespidae are aggressive and easily provoked. Individuals who are allergic to vespid venoms are often allergic to more than 1 species. In a survey of several hundred serum samples, 80% of yellow jacket allergic patients were also sensitive to hornets. Approximately 30% to 50% Hymenoptera venom sensitive patients are sensitive by skin test to honeybee venom and yellow jacket venom.³⁷

Three prominent allergens of yellow jacket venom are phospholipase A 1 (Ves v 1), hyaluronidase (Ves v 2), and antigen 5 (Ves v 5). In 2013, vitellogenin (Ves v 6), a 200-kDa molecular weight allergen, was identified,³³ but its function is unknown. Paper wasp also has 3 prominent allergens: phospholipase A 1 (Pol e 1), hyaluronidase (Pol e 2), and antigen 5 (Pol e 5). There is high IgE cross-reactivity among Hymenoptera allergens, which appears to be selective for protein domains, rather than carbohydrate determinants shared by honeybees and Vespidae species.³⁸

A method of vespid allergen extract manufacturing begins with collection of live insects from their nests by trapping at the entrance or by using a low-pressure vacuum system. For aerial nests, collection bags are slipped under the nests, and nests are detached from the attachment and immediately sealed. After collection, the insects are collected and frozen. The frozen insects are then thawed for dissection and removal of the venom sac. The collected venom sacs are crushed and filtered to purify the venom then freeze-dried and stored in a freezer. (For additional information on venom

collection processes, see <http://www.alk-abello.com/CA/services/fightthecause/Documents/Spring.2009.VenomNewsletter.pdf>).

Relatively pure forms of venom proteins can be obtained by milking individual insects, but this technique is tedious and inefficient for commercial use.

In the United States, vespid venom protein extracts containing various species of yellow jackets, wasps, and hornets are standardized. For vespid extracts, commercial manufacturers report the unitage of venom protein in micrograms per vial and the hyaluronidase and phospholipase A enzyme activity in units per milliliter.

Fire Ant and Harvester Ant

Fire ants (Formicidae) fall under another larger group of stinging insects. The most prevalent stinging ants are fire ants or imported fire ants (*Solenopsis invicta*), which are known for their aggressive attacking behavior. Fire ant nests are usually underground or under wooden logs or rocks, but they are often exposed as mounds as tall as 18 in (approximately 46 cm). There are approximately 20 American species of *Solenopsis* that have infested more than 310 million acres of land, most of which are in the Southeastern United States.³⁹ Fire ants bite to grip their victim and sting repetitively until the venom sac is empty. In some areas of the Southeastern United States, as many as 30% to 50% of people are allergic to fire ant venom.^{39,40} In 1989, a survey conducted by the Fire Ant Subcommittee of the American Academy of Allergy, Asthma, and Immunology reported 83 fatal and 2 near-fatal fire ant sting reactions.⁴¹

Fire ant venom is known as solenopsin, an alkaloid that burns and induces local erythema and the formation of sterile white pustules. More than 90% of the fire ant venom consists of piperidine alkaloids, and the remaining 10% is an aqueous solution of proteins. Potent allergens in fire ant venoms are Sol i 1 (phospholipase A/B), Sol i 2 (pheromone binding proteins), Sol i 3 (antigen 5 pathogenesis related protein), and Sol i 4 (a protein of unknown function).⁴²

Skin reactivity to extracts derived from whole bodies with venom and whole bodies alone correlate because the whole-body extract contains enough venom to make a clinically useful reagent.⁴³ As is the case for all nonstandardized extracts, fire ant (*Solenopsis invicta* and *Solenopsis richteri*) whole-body extracts are sold in the United States without any measure of potency. The concentration is labeled as 1:10 wt/vol.

Harvester ants belong to the genus *Pogonomyrmex*. There are more than 70 species of harvester ants, most of which inhabit arid areas in North and South America.⁴⁴ Harvester ant venom is rich in phospholipase A2 and B, hyaluronidase, acid and alkaline phosphatases, lipases, and esterases.^{45,46} In 2005, Klotz and colleagues⁴⁷ reported that many species other than the imported fire ants may induce local and systemic allergic reactions. In 1977, Pinnas and colleagues⁴⁸ described 8 patients who are allergic to harvester ants. Four patients had systemic reactions, 1 of whom had laryngeal edema and wheezing. The other 4 patients had large local reactions.

Biting Insects Allergens

Salivary proteins injected during insect bites cause local swelling but rarely induce systemic allergic reactions.

Kissing Bugs (*Triatoma protracta*)

Kissing bugs or Triatominae are carriers of the protozoa *Trypanosoma cruzi*, the causative agent of Chagas disease. Kissing bugs are found extensively in arid areas of the Southwest United States. The bites are painless and are often undetected, but salivary allergens induce erythematous plaques or other cutaneous allergic reactions. In a population-based survey conducted in 2012 in rural areas of California, 13% of individuals had symptoms related to

allergic reactions to Triatominae bites. *Triatoma* allergens are found in salivary fluid, and salivary glands are used as the source of allergens. The major allergen of *Triatoma* saliva is procalin.⁴⁹

As with all biting insects,³ most kissing bug reactions are local. However, systemic reactions, including urticaria, angioedema, wheezing, and laryngeal edema, have been reported.⁵⁰

Mosquito

Mosquito (*Aedes*, *Culex*, and *Anopheles* species) allergy is reported worldwide. Mosquitos can sensitize individuals through inhalation and bites, which may induce local and systemic allergic reactions. Most commonly, cutaneous reactions are at the bite site and consist of immediate wheal and flare reactions or delayed erythematous papules that peak at 24 to 36 hours and resolve in days or weeks. Among 70 individuals in Thailand who are allergic to *Culex quinquefasciatus*, only 2 reported systemic allergic reactions, which were limited to 1 episode each of urticaria or angioedema.⁵¹

Saliva and bodies of mosquitos belonging to genera *Aedes*, *Culex*, and *Anopheles* contain up to 19 allergens, ranging in molecular weight from 16 to 95 kDa. Whole-body extracts of *Aedes* species and *Culex* species have been used for serum IgE-binding studies. Serum IgE antibodies bind to 32- and 58-kDa proteins in *Aedes* extract. Aed a 1 is a 68-kDa protein from *Aedes aegypti*.⁵² *Culex* extract contains allergens that are 22, 32, 35, and 58 kDa.^{51,52} Similar to cockroach, the response to mosquito allergens is heterogeneous, and there are no dominant allergens that can define the potency of allergenic extracts.

Commercially available mosquito extracts are made of whole bodies that contain salivary and nonsalivary proteins. Occasionally, mosquito salivary extract is also used for skin prick testing. To obtain mosquito saliva, living mosquitoes are captured and their proboscises are inserted into collection tubes. For research-oriented studies, salivation is stimulated by applying malathion to thoraces. The saliva is collected, pooled, and lyophilized.⁵¹

Less Common Insect Allergies

Occupational asthma has been reported among laboratory workers who breed insects, such as grain weevils and locusts.⁵³ Burge et al⁵³ reported that among 110 industrial workers in a locust-breeding facility, 55% of workers had positive skin reactions that coincided with their allergic symptoms, and sensitization was related to the duration of exposure.⁵³ To our knowledge, there are no commercial products in the United States or elsewhere for the diagnosis or treatment of allergy to grain weevils or locusts.

Insect Allergen Extract Preparation and Control

It is critical to understand that insect extracts are compositionally complex and often variable mixtures of allergenic and nonallergenic components. Several steps in their manufacture may contribute to variability among products, for example, whether insects are cultured or harvested from their natural habitats and variation of cultures according to what the cultured insects are fed. In addition, time, harvest, and sex proportion of the insects may affect quantities of individual allergens. Finally, the equipment used for extraction, the extraction process, and storage conditions may also contribute to variability among allergen extracts.⁵⁴

Like many other allergen extracts, a stepwise approach is taken for the production of an insect allergen extract.⁵⁴ Below are basic steps that are often used for preparation of allergenic extracts in the United States. These methods do not apply to venom extracts. No proprietary information is included in this section.

Source Material

Selection of source material depends on insect type and has been discussed under each insect section. In some instances whole bodies are used, whereas in others specific body parts are used as allergen source. In the case of standardized venom protein products, only venom sacs are used. Occasionally, frass (dried up feces) and other secretions are also used along with whole body. For biting insect allergen extracts, salivary glands are considered the source material. The source materials are verified for identity and purity before processing for the extraction as indicated below.

Extraction

Most but not all of the source materials are washed and defatted (typically with acetone) before extraction. Defatted source material is sieved, milled, and/or powdered, as appropriate, and allergens are extracted from the source material in a buffer and at a ratio commonly expressed as weight to volume or dry weight in grams per milliliter. Buffers (composition and pH), timing, temperatures, and weight to volume ratio may vary among manufacturers.

Clarification and Sterile Filtration

After extraction from the source material, the resulting extract is clarified to separate solids from the extract. Typically, a series of graded filters is used for clarification. Other techniques, such as dialysis and centrifugation, may also be used. Finally, the extract is filtered through a 0.2- μ m filter for sterilization.

General Testing and Release

The final nonstandardized extracts are tested by the manufacturer according to their specifications in their license and labeled.^{54,55}

Variability among different manufacturers may be minimized by keeping the manufacturer of the source and culture conditions consistent and by large-scale preparation of source material. Carefully identified sources with appropriate specifications can improve consistency among allergen extracts. Appropriate measures to prevent denaturation during extract preparation and storage to ensure intact and biologically active components should be developed, and manufacturers should adhere to those standard operating procedures. Avoiding elevated temperatures and extreme pH and ionic conditions will help preserve proteins in their native forms. Use of low-molecular-weight cutoff filters (<5,000 Da) may be used only when low-molecular-weight substances are not important.

In the United States, the Center for Biologics Evaluation and Research maintains dry venom for lot testing and potency of honeybee venom. Manufacturers are provided this reference material to test potency of their lots before commercial release. European manufacturers maintain their own in-house-reference standard for batch to batch consistency of allergen extracts. The in-house-reference standard is characterized by *in vitro* methods, and biological activity is determined by skin prick test *in vitro* methods in allergic patients.⁵⁶ An allergen extract batch manufactured for commercial use is compared with the in-house-reference standard with qualitative and quantitative laboratory tests before batch release.⁵⁷

Conclusion

The steps for extracting allergens from source materials are relatively similar among the various products. However, methods of obtaining source materials may vary among manufacturers and their suppliers. Consequently, allergen extracts may qualitatively or quantitatively vary among manufacturers or even among lots from the same manufacturer.

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