

Case report

Identification of important allergenic proteins in extracts of the granary weevil (*Sitophilus granarius*)

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This paper describes the identification of important allergens from the granary weevil (*Sitophilus granarius*) (Sg). Sera from Danish bakers whose skin prick tests were positive to extracts of Sg were screened for IgE against Sg extracts. We found that 54% ($n = 66$) had elevated levels of IgE (RAST classes 1–3, by luminescent immunoassay) against whole-body extracts of Sg. The specificity of patient IgE was investigated in an inhibition-dot immunoblotting assay. IgE binding was inhibited in all sera but two, thus indicating that the patients' IgE was indeed specific for the Sg extract. In crossed immunoelectrophoresis, 23 different proteins were identified. All RAST-positive sera were investigated in crossed radioimmuno-electrophoresis. At least 11 proteins in the Sg extract were capable of binding IgE. All individual sera reacted with at least four different proteins. The two most prominent allergens bound IgE from 88% and 100%, respectively, of the patients. These two are considered to be the most important allergens from Sg, and will be useful as markers in environmental immunochemical assays to detect allergens in samples from bakeries, grain stores, etc.

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The granary weevil, *Sitophilus granarius* (Sg) (Coleoptera: Curculionidae), is a common pest in stored grain. The first indication of its significance as a cause of IgE-mediated allergy came from the UK, where studies indicated that Sg caused allergy in people working at an infested plant (2, 7). After these studies, the problem did not receive any attention until a Swedish study which concluded that the granary weevil played a role in farmers' allergy (3). Recently, a German study has shown that another member of this genus, the rice weevil, *S. oryzae*, caused IgE-mediated allergy among people living in a former grain store (6). This study found several IgE-binding components, ranging from 30 to 70 kDa, by SDS-PAGE and immunoblotting. The same study observed cross-reactivity to Sg. In a previous study at our laboratory, Danish bakers were skin-prick-tested with extracts of Sg, and a part of the population showed positive reactions (results provided by L. Nüchel-Petersen, to be published elsewhere). In order to delineate baker's occupational

allergy, one must, of course, consider the great variety of other allergy-causing sources to which bakers are exposed in their environment beside the granary weevil. Flour contains allergens originating from the grain, as previously shown by other researchers (15), who have detected allergens in the different fractions of wheat extracts (i.e., the albumin, gliadin, and glutenin fractions) with sera from allergic bakers. Amylases and cellulases of microbial origin added to improve baking have also been shown to cause allergy among bakers (1, 9).

Bakers may meet a number of allergenic challenges from pests in their working environment such as domestic/storage mites (i.e., *Lepidoglyphus destructor*, *Tyrophagus putrescentiae*, and *Acarus siro*), house-dust mites (*Dermatophagoides* spp.), and cockroaches. Many investigators have shown that these animals are the source of several potent allergens (4, 11). Thus, allergens from Sg will probably be only a part of the allergy-causing sources to which bakers are exposed in their environment.

This study demonstrates IgE-binding components in whole-body extracts of Sg by the dot-immunoblotting technique, the luminescent immunoassay (LIA), and crossed immunoelectrophoresis. The IgE source was sera from atopic Danish bakers who had participated in the above-mentioned study at our laboratory.

Material and methods

All chemicals used were reagent grade or better.

Preparation of extracts of S. granarius

Cultures of *S. granarius* were obtained from the Danish Pest Infestation Laboratory (Lyngby, Denmark). Adult weevils were taken from a 2-month-old culture and killed by freezing (-20°C). The weevils were cleaned by ultrasonication in alcohol. The extraction was performed after carefully crushing the weevils in a mortar. As extraction buffer, we used 0.125 M $(\text{NH}_4)\text{HCO}_3$. All extractions were performed at 10% w/v at 5°C overnight in a shaker. The extract was subsequently centrifuged for 30 min (27 000 g). The supernatant was dialyzed against 50 volumes of 5 mM $(\text{NH}_4)\text{HCO}_3$ for 3/2 days, followed by dialysis against distilled water for 1/2 day. Dialysis was performed at 5°C with a 10-kDa cut-off dialysis membrane. Finally, the material was freeze-dried. Typical yield was 50 mg dry weight/gram weevils extracted. The protein concentration in redissolved extract was determined by the method of Lowry. The extract was used in different analyzing techniques in order to reveal the allergenicity and to identify allergens.

Patient population

In the study, we used sera from 66 Danish bakers with a positive skin prick test (SPT) to extracts of Sg. The 66 patients were all men, their ages ranged from 18 to 66 years, and all had participated in screening for occupational allergies among 576 bakers in Copenhagen County, Denmark. None had been undergoing immunotherapy prior to the investigations. As control, we used sera from nonatopic Danish bakers who had participated in the same study.

Dot immunoblotting

Serial dilutions of Sg extracts dissolved in 0.1 M NaCl, 15 mM NaN_3 , were bound to nitrocellulose strips (NC), 0.45 μm (Millipore) by application of 10 μl of solution on the NC, and were allowed to dry at room temperature (RT). The amount of protein bound ranged from 25 to 0.25 μg . The NC strips

were blocked with Tris/NaCl and 2% Tween 20, pH 10.3, and washed with Tris/NaCl, 0.05% Tween 20. The strips were incubated overnight with human sera diluted 50 times in PBS, 0.5% BSA, and 0.05% Tween 20, pH 7.4, from either SPT-positive patients or the control population. After washing four times with PBS, 0.05% Tween 20, the strips were incubated with 50 000 cpm ^{125}I -labeled antihuman IgE antibodies (Phadebas RAST, Kabi-Pharmacia, Sweden) overnight at RT. The antibodies were diluted in PBS and 0.5% BSA. Finally, after four washings in PBS and 0.05% Tween, the strips were used in autoradiography, in which they were allowed to expose radiographic films in Kodak X-Omatic cassettes with intensifying screens at -80°C for 8–10 days. The films were then developed.

Inhibition-dot immunoblotting

Dot-blot-positive sera diluted 80 times were mixed with different amounts of Sg extracts, 100, 10, 1, and 0.1 μg in total volume of 1 ml, and allowed to incubate at RT for 3 h. The mixture was transferred to small tubes containing pieces of NC (2×0.5 cm), where dots of 20- and 2- μg extracts were adsorbed as above. After overnight incubation at RT, the NC strips were washed four times in PBS. ^{125}I -labeled aIgE (50 000 cpm) was added, and the strips were incubated overnight at RT as described above. After four washings, the strips were dried and used for autoradiography (8–10 days).

Luminescent immunoassay (LIA) analysis of patient sera

Although the screening for IgE in patients by dot-immunoblotting assays gave positive results, the method did not give any semiquantitative measurement of the patient IgE levels (e.g., RAST class). Therefore, we chose to perform specific IgE measurement by LIA. Sg extracts were covalently coupled to paramagnetic microscopic particles. The magnetic particles were used as solid phase in polystyrene tubes. The magnetic particles were incubated with patient serum and subsequently with monoclonal antihuman IgE coupled to chemiluminescent acridinium ester (ALK, Hørsholm, Denmark). Bound IgE was revealed in a luminometer (Magic Lite analyzer, Ciba Corning Diagnostics).

Preparation of rabbit antibodies against Sg extracts

Rabbits were immunized essentially as previously described (5). Briefly, three rabbits were immunized with 3 mg/ml extract emulsified with Freund's incomplete adjuvant. The rabbits were given 0.1 ml emulsified antigen subcutaneously four times at

2-week intervals. Thereafter, the immunizations took place at 1-month intervals. Antisera were obtained by monthly bleedings, 12 days after previous immunization. Antibodies were purified from serum by ammonium sulfate precipitation and stored in 0.9% NaCl, 15 mM NaN₃, at 4°C.

Crossed immunoelectrophoresis (CIE) and crossed radioimmuno-electrophoresis (CRIE)

CIE was performed by previously described methods (14). In CIE, the first-dimension electrophoresis of 100 µg Sg extract was run at 4 V/cm for 45 min. In the second dimension, separated Sg extract was electrophoresed into purified polyclonal rabbit antibodies (concentration of 5 µl/cm²) and run at 150 V total for at least 16 h. In CRIE, dot-blot/LIA-positive sera were applied to cold-dried plates after CIE. The optimal amount of serum was 400 µl in 8 ml sodium phosphate buffer per plate. After incubation with serum, the plates were washed and incubated with 250 000 cpm ¹²⁵I-labeled rabbit-antihuman IgE (Phadebas RAST). After overnight incubation, the plates were washed and autoradiographed for 10 days at -80°C with intensifying screens.

Results

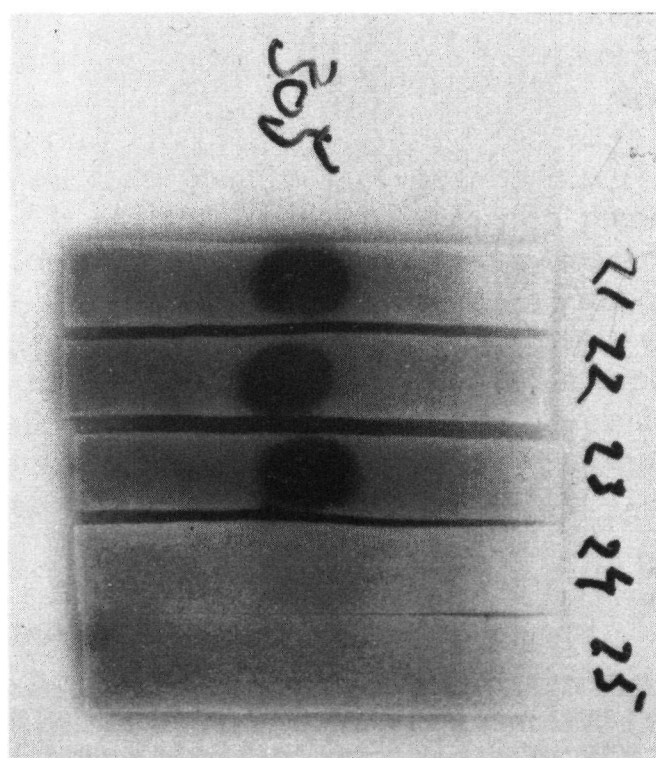
Screening of anti-Sg-IgE-containing sera by dot immunoblotting

The screening of patient sera by dot immunoblotting showed that 40% (*n* = 66) of the sera contained elevated levels of IgE. Most of the positive sera had strong binding even to the weaker dilution of the extract; that is, when 0.25 µg extract was immobilized on the NC. The binding of IgE and radiostaining were evaluated visually. As negative control, we used serum from 20 nonatopic Danish bakers. None of the control sera reacted to Sg extracts.

All dot-blot-positive sera were used in inhibition dot-immunoblotting experiments to verify the specificity of the sera. All autoradiograms were evaluated visually, and a representative serum can be seen in Table 1 (insert). The inhibition followed a dose-response curve (evaluated visually) over 1 decade of concentration increment for each individual serum. The concentration at which the inhibition took place differed among the individual sera. In all sera, the inhibition was total at an inhibitor concentration of 100 ng/ml. The results indicate that the IgE response was indeed specific for components in the Sg extract (Table 1).

Table 1. Number of sera inhibited by increasing concentrations of free granary weevil extracts. Insert shows inhibition of typical serum (subject 305). In all cases, inhibition followed dose-response curve over 1 decade of concentration increment (on insert from 1.0 to 10 µg/ml). Nos. 21–25 on insert indicate increasing levels of inhibitor, i.e., from 0 to 100 µg/ml

Concentration of inhibitor µg/ml	No. of inhibited sera
0.0	0
0.1	1
1.0	8
10.0	22
100.0	24



Luminescent immunoassay

The LIA experiments were done to obtain a semi-quantitative measure of patient IgE level against Sg extracts. All baker sera ($n = 66$) were used in these experiments. In this system, 34 (52%) sera showed elevated levels of IgE against the Sg extract. Positive sera showed RAST classes ranging from 1 to 3, with the same binding pattern as seen in dot-immunoblotting experiments; that is, the highest RAST classes also gave the highest grade of radiostaining. The latter showed that IgE-binding epitopes on Sg protein were not impaired by either of the two different immobilization techniques (adsorption on NC and covalent binding to magnetic particles).

Antigens in extracts of Sg

The CIE showed at least 22 precipitins when stained with Coomassie brilliant blue (Fig. 1); 100 μ g extract was then separated into 150 μ l purified rabbit-antibody solution. Three antigens migrated toward the cathode and the rest toward the anode. Some of the antigen-antibody precipitin complexes were intensively stained, whereas others were weakly stained. One extra precipitin line was revealed only by CRIE (denoted Ag 11; see below). To test whether any of the precipitins originated from the culture medium, we did experiments in which extracts of culture medium (whole wheat grains) were used as antigenic solution in CIE. It was not possible to detect any precipitin lines when 40 or more μ g wheat protein was electrophoresed against 150 μ l purified anti-Sg extract antibodies, and it was concluded that

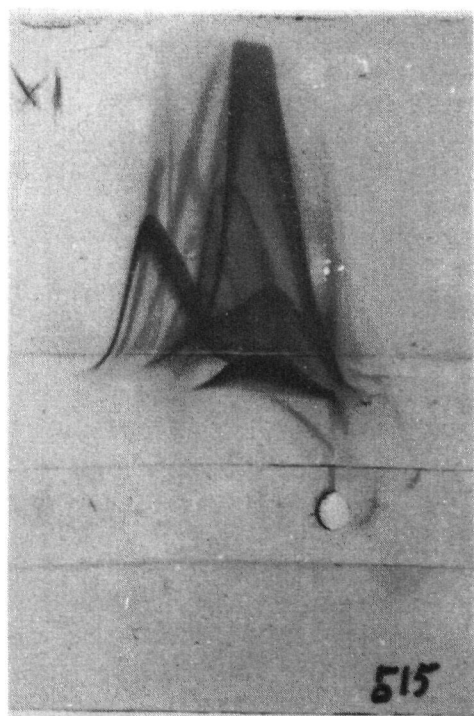


Fig. 1. CIE with granary weevil extract resulting in at least 22 Coomassie-stained precipitins.

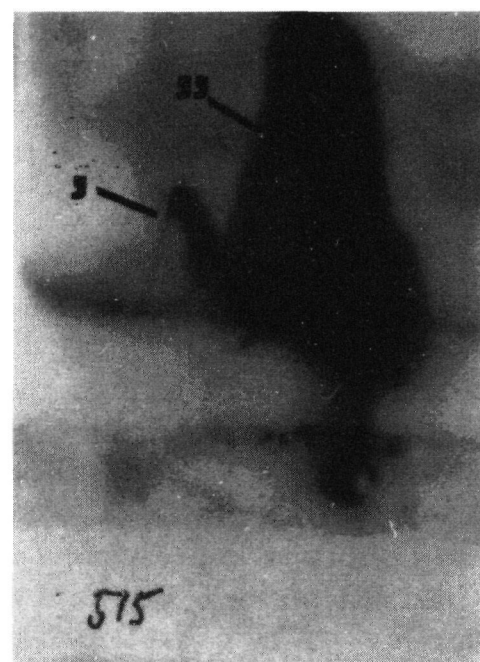


Fig. 2. Autoradiogram showing CRIE with representative serum. Most prominent allergens are (') Ag 1 and (") Ag 4.

the medium did not produce any artifactual precipitins in the experiments.

Allergens in extracts of Sg

Allergens from Sg were identified by careful inspection of CRIE autoradiograms. A typical CRIE autoradiogram is shown in Fig. 2. The autoradiograms showed that at least 11 (denoted Ag 1–11) proteins in the Sg extract bound IgE from Sg-allergic bakers. Ag 4 seemed to be the most powerful allergen, being recognized by IgE from all 34 patients (100%). Ag 1 was recognized by 30 patient sera (88%). Ag 2, Ag 6, Ag 8, and Ag 9 were recognized by 22 (64%), 19 (56%), 18 (53%), and 16 (47%), respectively. The rest were bound by less than 25% of the sera. Ag 11 was seen only in CRIE and did not stain with Coomassie brilliant blue. All sera recognized at least

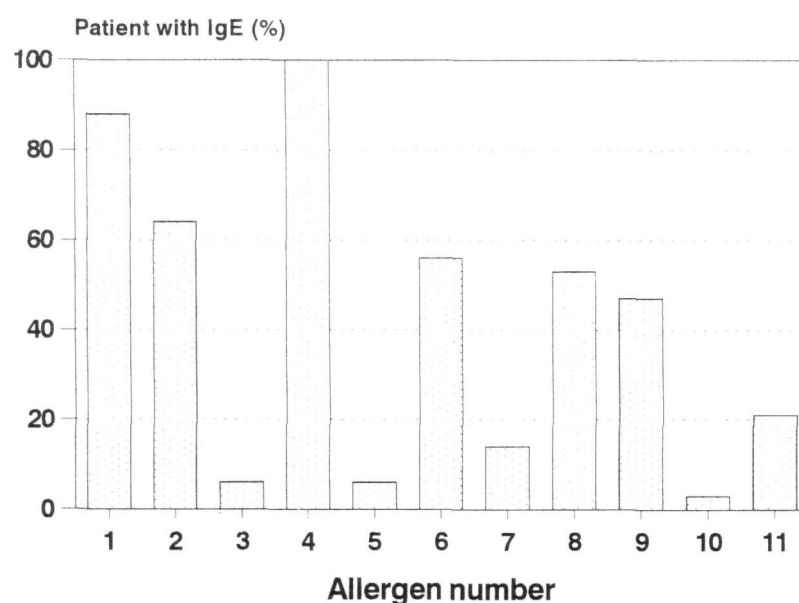


Fig. 3. Individual patient IgE against different allergens in extracts of granary weevil.

three allergens in the extract. The IgE titer against the same allergens varied from patient to patient. IgE recognition is summarized in Fig. 3. The allergens denoted Ag 5 and Ag 6 could be the same allergens, but, at present, it is not possible to distinguish between the two precipitins whether radiostained or Coomassie stained. The two allergens recognized by more than 85% of the sera are considered to be the most important allergens of the granary weevil.

CRIE with 10 individual sera from nonatopic bakers was done as a control. None of the sera used showed significant IgE binding (data not shown).

Discussion

For production of a good and pure extract of the granary weevil, it is important to have a good cleaning procedure. The surface of the weevils is covered by a thick layer of wax, which contains fecal particles and dust particles from the media, and there even may be contaminating mites. To break the wax layer, we used ultrasonication in ethanol, which indeed removed the layer and contaminating particles within it. By light microscopy, it was possible to determine when the animals were cleaned. It was necessary to use ultrasonication for about 30 min, preferably at intervals to avoid excessive heating of the weevils. Immunoelectrophoretic studies with an extract of uncleaned weevils did not reveal any extra proteins to be considered as potential allergens.

The extract produced contained at least 22 different antigens of different immunogenicity as revealed by CIE. One additional allergen was seen only in CRIE. Therefore, the total number of antigens was 23 with the rabbit antiserum used in this study.

We have shown that 54% of the SPT-positive patients had elevated serum IgE against proteins from the granary weevil. The differences between the results of two screening systems (dot immunoblotting and LIA) are probably due to different sensitivities in the assays.

Because of its metabolism, Sg can multiply only in whole grain; therefore, it is not found in significant numbers in bakeries. This fact suggests that the allergy-causing components that appear in stored grain persist after processing (e.g., cleaning and milling) of the grain. Thereafter, bakers are exposed to the weevil allergens, together with other dust and flour allergens, in their environment. Since the present results suggest that the granary weevil might be of clinical relevance as a source of allergen in the baker's environment, other grain-handling workers (e.g., millworkers, farmers, etc.) may also become sensitized. A recent study (3) of a Swedish farming population showed by RAST that only 1.1% of the farmers were allergic to Sg. A possible reason for

this difference might be that the weevils and their larvae live most of their lifetime inside the grains, and that the allergens of the adult weevils and larvae are liberated primarily during the milling of the grain. This would make Sg allergens a greater risk for workers handling the flour than for those handling the grain. However, the present results do not provide any clear evidence for occupational Sg allergy, and further epidemiologic studies are needed to establish whether or not Sg allergy is an occupational allergy.

As for baker's allergy, it is evident that many other factors in the baker's environment beside Sg cause allergy. Flour components (e.g., wheat or other grain proteins), baking-improving additives (e.g., fungal enzymes), and bakery-infesting pests (mites and other insects) have all been shown to cause allergy (5, 9, 11, 15). Wheat components and enzymes have been proved to be related to baker's allergy, while the relation between baker's allergy and infesting pests remains unclear (12, 13).

By CRIE, we found that the Sg extracts contained 11 allergens. Six allergens were considered to be potent as they reacted with at least 50% of the individual patient sera. Two allergens were very potent; that is, they reacted with at least 88–100% of the sera. Both produced a high grade of radiostaining after 10 days of autoradiography. The allergens identified in the present work seem to be the most important allergens of the granary weevil, when CRIE is used as identifying system. The two allergens will be suitable as markers in environmental assays to measure allergen contamination of grain stores, mills, flour, and bakery dust.

A polyclonal antibody-based ELISA has been developed in the USA, with myosin from the field cricket, *Acheta domestica*, as immunogen. Insect myosin is very similar throughout the different species, and has resulted in very species-unspecific (among insects) antibodies. This assay detects, among others, myosin from Sg, and is used to measure pest contamination of grain in storage (8, 10). However, since this assay is unspecific, there is a need for an Sg-allergen-specific assay, based on antibodies from animals immunized with the important allergens from Sg. An allergen-specific assay would allow studies of the relationship between exposure and allergy development to the granary weevil, and determine allergen persistence throughout the processing of grain and production of cereals.

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