University of Nebraska Lincoln

FOOD ALLERGY RESEARCH AND RESOURCE PROGRAM
Department of Food Science & Technology

23 March, 2018

Subject: Re-review of the potential allergenicity of the Green Fluorescent Protein family Alkane that was entered into AllergenOnline.org database in January, 2018.

Dear AllegenOnline.org user:

Thank you for asking about the validity of the Akane sequences that we entered into AllergenOnline.org in January, 2018, version 18 of our database. There are four closely related sequences that are from 89% to 99% identical, all entered in the GenBank public database as "Novel Allergenic Proteins" from the Octocoral species *Scleronephthya gracillima*, by authors of the publication (Kato et al., 2017, Luminescence 32(6):1009-1016).

Our original review of the publication by Kato was based on the process we described in 2016 for the generation and curation of our AllergenOnline.org database. However, this is one of the instances when the panel of experts was a bit divided in the original review. I reviewed our archival notes on version 18 and found that no one called this a clear "allergen", and there was grading by some that the protein was not even "putative" within our definition. Therefore I asked the rest of the panel (7 other experts) to join me in a re-review of the Kato et al., 2017 paper and our decision. I have described the details of the information in the Kato publication here, and the final decision that the group came to following the re-review, on 19 March, 2018.

Review points:

1. Only one publication can be found in PubMed of allergy to the source organism, *Scheronephthya sp*. That is the one by Kato et al., 2017.
2. Kato et al., described conjunctival, dermal and asthma disease symptoms in some fishermen and workers who process mollusks and in lobster fishermen. They also described testing guinea pigs with extracts of a related red octocoral organism.
3. Kato et al., experimental details.
    a. Serum donors included a panel of an unlisted number of patients who are lobster fishermen from the Pacific coast of Miyazaki, with the range of symptoms (conjunctivitis, dermatitis or asthma), and they pooled their sera. They also had a pool of "healthy controls".
    b. Kato et al., collected octocoral samples of *S. gracillima* and extracted proteins in 10 mM sodium phosphate buffer, then concentrated proteins by precipitation with ammonium sulfate, then dialyzed to remove ammonium sulfate.

1901 North 21st Street / Nebraska Innovation Campus / PO Box 886207 / Lincoln, NE 68588-6207
Richard E. Goodman, PhD, rgoodman2@unl.edu Office phone (402) 472-0452; Cell (402) 417-5549

c. Kato et al., performed partial purification of the fluorescent protein(s) using gel filtration and ion exchange chromatography, following the protein with UV absorbance detection.
d. Kato et al., separated the semi-pure mixtures by SDS-PAGE and transferred proteins to PVDF membranes for immunoblots with pooled patient and control sera. The western blots were blocked with non-fat dry milk, and after serum samples were incubated and washed, they used polyclonal goat anti-human IgE coupled with horse radish peroxidase (HRP). The HRP was detected with chemiluminescence (ECL Plus), with exposure of X-ray films. There was faint binding to a 27 kDa band, not to control serum pool by 1D immunoblots. There was stronger detectable band to a 22 kDa band by control and patient pools (figure 2).
e. Paragraph 2.6 in Kato et al., seems out of place. They describe selection of IgE from patient pool by immunoblotting onto a crude extract, then using that captured sera as primary antibody. It is not clear if that was used in the first 1D immunoblot, or only in the 2D immunoblot.
f. Section 2.8 describes trypsin-digestion of the proteins, 22 kDa, 27 kDa and 45 kDa. They only describe two peptides (one has an ambiguity "I" vs"L" as part of the 27 kDa protein (Table 1), the other, 10 aa peptide is shown in Table 1 and is in Table 2 as peptides from the 8 spots in the 2D gel (Figure 6) which shows immunoblots of the pooled "allergic" sera, and the pooled "control" sera. Interestingly the 10 AA peptide was seen in all 8 spots from 27 kDa and the 22 kDa spot. No peptides are identified from the 45 kDa spot.
g. Section 2.9 describes cDNA cloning by RACE, starting from the Poly A selected RNA. Unfortunately there is no description of the starting sequence for RACE.
h. Figure 5 shows 1D immunoblots of "raw" 22, 27 and 45 kDa proteins with immunoblots without absorption ((b) and with absorption (c), P-1, not P-2, of sera bound or not to raw extract. Kato et al., attribute the apparent lack of binding to 27 kDa protein in (c) as indicating that the only allergenic protein if from 27 kDa protein.
i. Figure 6 (b) shows very light spots 4, 6 and 7 at 27 kDa with patient sera, but a very intense two spots at 22 kDa that they dismiss. Clearly from stained gel (a), the two spots are most abundant. However, the spots 6 and 7 in stained gel are fairly abundant based on staining. Yet immunoblots show only faint "ghost" spots (b) and lighter in (c) with control sera on spot 7.
j. The results describe interesting fluorescence behavior of the proteins showing emissions for green and red proteins when stimulated with UV.
k. Kato et al., have not demonstrated clear IgE binding to proteins that they claim to have cloned. They also show only light apparent binding in a 2D immunoblot to spots 4 and 6. They have not shown sequences that correspond to all of the blots, not have they demonstrated any differences that would explain why spots 4 and 6 should be "allergens", except apparent light immunoblot patterns.

SUMMARY. In our first review of the data of the "Akane" proteins presented by Kato et al., 2017, some of the panel thought there was sufficient evidence to suggest that the protein(s) described by Kato et al., 2017, could be considered "putative allergens" and included in our version 18 database. However, as we have gone through a second round of review and looked at their publication a second time in great detail, the complete panel of eight allergen experts (listed below), have concluded that there is not sufficient evidence to call the protein(s) even putative allergens. The authors (Kato et al) have speculated on dimers of the protein that have not been demonstrated for this protein, and they have speculated that their results demonstrate allergenicity. However, the requirements of our classification scheme presented in Goodman et al., 2016 has not been sufficiently demonstrated to approve the four cDNA sequences listed as Accession numbers BAW321535.1, BAW32536.1, BAW32537.1 and BAW32538.1, as "putative" allergens. Certainly they lack proof of biological activity of "allergens" that would require not only specific IgE binding, but also biological activity of allergenicity (basophil activity, skin prick test reactivity or other in vivo challenge positive reactivity).

As a panel, we have unanimously agreed to remove these four sequences from the AllergenOnline.org database since the only data of "possible" allergenicity is that presented by the Kato et al., 2017 publication.

REVIEW PANEL:

1. Joe Baumert, Ph.D.,
   University of Nebraska, Lincoln, NE, USA
2. Barbara Bohle, Ph.D.,
   Medical University of Vienna, Vienna, Austria
3. Motohiro Ebisawa, M.D.,
   National Sagamihara Hospital, Sagamihara, Japan
4. Fatima Ferreira, Ph.D.,
   University of Salzburg, Salzburg, Austria
5. Richard E. Goodman, Ph.D.,
   University of Nebraska, Lincoln, NE, USA
6. Joerg Kleine-Tebbe, MD, FAAAAI., (member since 2016)
   Allergie- & Asthma-Zentrum Berlin Westend, OPD Hanf, Ackermann & Kleine-Tebbe, Berlin, Germany
7. Steve L. Taylor, Ph.D.,
   University of Nebraska, Lincoln, NE, USA
8. Ronald van Ree, Ph.D.,
   Academic Medical Center, Amsterdam, The Netherlands

References:

Kato Y, Jimbo M, Sakakibara Y, Onizuka R, Takahashi T, Matsuhashi S, Mita H, Amada K, Imahara Y, Tanabe K, Toda A, Kamiya H. 2017. Characterization of a novel allergenic protein from the octocoral Scheronephthya gracillima (Kuekenthal) that corresponds to a new GFP-like family named Akane. Luminescence. 32(6):1009-1016.

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Sincerely,

Richard E. Goodman

Richard E. Goodman, PhD, FAAAAI
Research Professor
Manager, AllergenOnline.org
Chair of the WHO/IUIS Allergen Nomenclature Sub-Committee
FARRP, Food Science & Technology
Food Innovation Center
University of Nebraska
1901 21st Street
Lincoln, NE 68588-6207
rgoodman2@unl.edu