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Safety assessment of the calcium-binding protein, apoaequorin, expressed by *Escherichia coli*

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Abstract
Calcium-binding proteins are ubiquitous modulators of cellular activity and function. Cells possess numerous calcium-binding proteins that regulate calcium concentration in the cytosol by buffering excess free calcium ion. Disturbances in intracellular calcium homeostasis are at the heart of many age-related conditions making these proteins targets for therapeutic intervention. A calcium-binding protein, apoaequorin, has shown potential utility in a broad spectrum of applications for human health and well-being. Large-scale recombinant production of the protein has been successful; enabling further research and development and commercialization efforts. Previous work reported a 90-day subchronic toxicity test that demonstrated this protein has no toxicity by oral exposure in Sprague–Dawley rodents. The current study assesses the allergenic potential of the purified protein using bioinformatic analysis and simulated gastric digestion. The results from the bioinformatics searches with the apoaequorin sequence show the protein is not a known allergen and not likely to cross-react with known allergens. Apoaequorin is easily digested by pepsin, a characteristic commonly exhibited by many non-allergenic dietary proteins. From these data, there is no added concern of safety due to unusual stability of the protein by ingestion.

Keywords: Safety assessment, Apoaequorin, Allergenicity, Bioinformatic, Calcium-binding protein

1. Introduction

The calcium-binding protein apoaequorin, endogenous to the luminescent jellyfish *Aequorea victoria*, has been used for over 40 years as a research molecule in studies of intracellular physiology of the calcium ion. This small protein (22.3 kDa) possesses three calcium-binding sites of the EF-hand motif and belongs to the superfamily of calcium-binding proteins that facilitate signal transduction, gene transcription, enzyme activation, intracellular calcium buffering and in the case of conjugated form of apoaequorin, aequorin, and related proteins, bioluminescence (Deng et al., 2004, Heizmann, 1992, Deng et al., 2005 and Yáñez et al., 2012). When the apo-form of the protein is conjugated to its prosthetic group, coelenterazine, the resulting molecule, aequorin, luminesces upon binding the calcium ion *in vivo* or *in vitro* (Kendall and Badminton, 1998). Properly stimulated, cells containing aequorin release light showing where, when and how much free, cytosolic calcium ion is released from internal stores or across the cell membrane in response to stimuli (Snowdowne et al., 1984). This photoprotein has been used for *in vitro* and *in vivo* studies as an indicator molecule for understanding calcium's role in signal transduction, metabolism, development, and particularly, brain cell physiology (Grienberger and Konnerth, 2012 and Kendall and Badminton, 1998). Some calcium-binding proteins coordinate the role of the calcium ion in the cell; maintaining calcium homeostasis within finely tuned concentration limits to avoid toxicity while preserving calcium's influence on cell physiology.

Because of its unique commercial value, a large-scale manufacturing process has been developed to produce kilogram quantities of this protein for use in a number of applications pertinent to human health and well-being. The potential increased exposure of human and animal populations to this protein required a toxicological and allergenic analysis from regulatory agencies and industrial best-practice standards. An extensive 90-day subchronic toxicology study was performed in Sprague–Dawley rats to test the oral route of delivery of the purified protein (Moran et al., 2013). There were no clinical pathology or histopathological changes in the animals receiving the protein and any changes of note were typical of those historically observed changes in the age and strain of rats used in the study. A No Observed-Adverse-Effect Level (NOAEL) for apoaequorin was determined at 2000× (expected human exposure) and an ADI safety margin of 666.7 mg/kg bwt/day was established, which was the highest dose tested.

The current study continues the risk assessment of the protein through bioinformatic and simulated gastric digestion tests to evaluate the potential allergenicity of this protein. Characterization of the expressed protein included N-terminal sequencing, molecular weight determination, and luminescent activity, which verified the identity and functionality of the protein. With the expected post-translational processing and the fidelity of the genetic expression construction confirmed, an allergenic risk assessment was performed. This included a protein sequencing analysis in an exhaustive bioinformatic assessment against allergen databases and an *in vitro* simulated gastric digestion of the protein. These approaches have proven invaluable as tools in assessing the potential allergenicity of genetically engineered proteins since they consider the primary amino acid sequence of known allergenic proteins that could pose a risk of allergy to those sensitized by other exposure and in evaluating the likelihood the novel protein might pose a high risk of sensitization or elicitation upon ingestion. Although the tests cannot prove an absence of risk of allergy, they have been deemed highly predictive of a protein's allergenic potential.

In vitro digestion in pepsin was performed to determine the level of resistance to protease cleavage under optimum pH conditions. Investigation of proteins that have been tested in this way suggest a strong positive predictive value that food allergens causing systemic reactions are relatively stable in the assay, while non-allergenic food proteins are typically digested relatively quickly (Bannon et al., 2002). Astwood et al. (1996) published the first use of *in vitro* pepsin digestion as an assay showing that many food allergens or protein fragments were resistant to digestion. Thomas et al. (2004) published a method standardizing the pH, purity of the protease, ratio of pepsin to test article, and purity of the test protein. We (Ofori-Anti et al., 2008) added an objective measure of the degree of digestion to improve the evaluation for assessing digestibility. However, although a correlation exists between allergenicity of dietary proteins and pepsin resistance (Astwood et al., 1996) some unstable allergens may be protected in the food matrix of the gut *in vivo* (Polovic et al., 2007). Since no single determinant has been found to be full proof for predicting allergenicity, international consensus has established a weight-of-evidence approach in assessing allergenic potential of new proteins (Codex, 2003).

2. Method and materials

2.1. Strain development

A synthetic gene for the protein sequence P02592 (UniProtKB/Swiss-Prot) was codon-optimized for expression in a prokaryotic cell line. The synthetic gene (Life Technologies) was subcloned into an expression cassette following standard molecular protocols for site-specific integration into the host strain, *Escherichia coli* BL21(DE3), and targeted secretion to the fermentation medium. Genomic insertion of the gene was performed by the homologous recombination method of Hamilton et al. (1989) with variations needed to target a non-essential operon of the host strain. Clones carrying only the expression cassette minus vector DNA were isolated using PCR for both the gene of interest and the antibiotic resistance gene. Isolated clones were verified by Southern blot analysis using radiolabeled probes of the antibiotic and gene of interest sequences (data not shown). A single clone was developed for industrial production of the protein and was named QB6.

The fidelity of the genomic insertion sequence was verified by DNA sequencing through PCR amplification using the primer pairs specific to the coding region; CAG TAT AGC GTC AAA CTG as the forward primer and TGG TGC TCG ACT TAT TAC for the reverse primer. A single band of the PCR product was visualized by agarose gel electrophoresis. The band was excised from the gel and was sequenced analysis in both directions (ACGT, Inc., Wheeling, IL).

2.2. Protein production and purification

Fermentation of the strain was performed by large-scale production processes and successfully produced multi-gram quantities of the secreted protein using lactose induction in a proprietary medium formulation. Clarification of the resulting fermentation broth by tangential flow filtration was followed by large-scale ion exchange chromatography. This resulted in a solution of the protein that was 90% pure. Acid precipitation with 10% acetic acid to a pH of 4.2 was followed by centrifugation at 2500*g* for 30 min. The supernatant was poured off, which aided in the removal of UV absorbing contaminants, leaving concentrated protein as the pellet. Resuspension of the protein (25 mM TRIS, 25 mM NaCl and 1 mM EDTA, pH 8.5) followed by gel filtration chromatography removed residual salts and contaminants. Pooled fractions from gel filtration were concentrated through tangential flow filtration using membranes of a nominal molecular weight cutoff of 5 kDa to obtain an approximate concentration of 20 mg/mL solution as determined by absorbance at 280 nm (A280) and a theoretical extinction coefficient for the apoprotein of 2.0. The protein was sterile filtered and kept frozen at −20 °C in buffer (25 mM TRIS, and 25 mM NaCl, pH 7.6), until use.

2.3. Protein characterization

N-terminal sequence of the first 30 amino acids of the recombinantly expressed apoaequorin was determined from samples purified from culture medium by SDS–PAGE and electroblotted onto a PVDF membrane. The protein bands were visualized by Ponseau S staining and N-terminal sequencing was completed at the Medical College of Wisconsin Protein and Nucleic Acid Facility.

The protein was further characterized by SDS PAGE (Novex Bis-Tris 4–12% gradient gels following the manufacturer's instructions – Life Technologies). The gel was stained with a commercial preparation of coomassie brilliant blue solution (Thermo Fisher Imperial Protein Stain) and destained overnight in distilled water. The destained gels were visualized in a Kodak Gel Logic 440 Image Station under white light transillumination. The image was captured and purity was estimated by densitometric analysis of the stained gel. This estimate was used in digestion studies of the protein.

Enzymatic activity of the protein was determined by regenerating the holoprotein, aequorin, and demonstrating calcium-binding activity through the chemiluminescence of the protein. Methods for the regeneration, luminescent activity, and measurements of photons followed Shimomura (1991) with minor modifications. Briefly, the assay was miniaturized for use in white, 96-well microtiter plate format to measure luminescence. Bioluminescence was measured on a Biotek luminometer plate reader controlled by the KC4 software (Biotek). Samples were discharged to produce blue light (462 nm) by the addition of 300 µL of assay buffer (20 mM calcium acetate in 10 mM TRIS, pH 7.6). Photons were collected over a three-second time period and maximum luminosities were averaged by the software for each dilution of each sample. The average relative luminous units were calculated for each sample. The results from eight dilutions were averaged for each sample and these data were validated by comparative analysis against the protein's kinetic accumulation during fermentation.

2.4. Allergenicity testing: Sequence search in silico

Potential allergy and cross-reactivity risk assessment was performed using methods typically used for food-safety evaluation as

prescribed by the Codex Alimentarius Guidelines (2003). The bioinformatics study was designed to compare the protein sequence of apoaequorin with those of known allergens in 2010 with version 10 of the AllergenOnline.org database (AOL v10) maintained by the Food Allergy Research and Resource Program (FARRP) at the University of Nebraska. This was repeated with version 13 of AllergenOnline.org on 5 December 2013 along with BLASTP searches of NCBI Protein database to evaluate possible matches to newly discovered allergens. The purpose of the search was to determine whether the protein could be identified as a known allergen (~95–100% match to an allergen listed in AllergenOnline.org), or was sufficiently similar to a known allergen (>35% identity over any segment of 80 amino acids) to suspect potential allergic cross-reactivity. The first search was performed using the FASTA3 algorithm (Pearson, 2000) with the default scoring matrix (BLOSUM 50) to evaluate overall alignment of each query sequence compared to all sequences in AllergenOnline, looking for matches of low *E*-score values (<1e-7) and greater than 50% identity as likely significant scores suggesting potential cross-reactivity.

Additionally, an 80-amino acid sliding window identity search was performed that systematically tests all possible 80-amino acid segments of apoaequorin with >35% identity to any known or putative allergen listed in the AOL database, using the same FASTA3 algorithm. Significant matches identified by either search would suggest a need to perform human serum testing using sera from specifically allergic donors to evaluate for potential IgE cross-reactivity (Codex, 2003).

Additional information considered in this study included a search of the protein sequence for any eight contiguous amino acid matches (8mer) to any known allergen in AOL. This step is required by some international regulatory agencies however, it has not been demonstrated to have a substantial predictive value for allergenicity. This step was included to meet our regulatory purposes.

The protein sequence was also searched against the non-redundant NCBI dataset using BLASTP with default values for BLOSUM 62 to evaluate homologs of the query protein to understand protein function and further screen for potential safety issues. This protein is in the family of Ca^{+2} binding proteins and is most closely related to similar proteins from other hydrozoans of the phylum Cnidaria with *E*-scores smaller than 10^{-40} and identities of >60% over the full-length. Additionally, a BLASTP search using the keyword limit of "allerg*" to any "keyword" allergen sequence in NCBI with an *E*-score smaller than 10^{-3} or a sequence homology of >35% identity was performed.

References in the PubMed database were also searched using the organism name (*A. victoria*) as well as family name (Aequoreidae) and the keyword "allerg*" to learn whether there is information to indicate whether the source organism is a common cause of allergy.

2.5. Simulated gastric digestion

The pepsin stability assay is one study in a "weight of evidence approach" intended to assess the potential allergenicity of genetically modified crops (Codex, 2003). The assay described by Astwood et al. (1996) was refined further; adding an objective measurement of the extent of digestion as described by Ofori-Anti et al. (2008). The assay was performed under standard conditions of 10 units of pepsin activity per microgram of test protein using a high purity form of pepsin from Worthington Biochemical, Corp., The mass ratio was approximately 3.6 mg pepsin per mg of test protein and other quality control measures as recommended by Ofori-Anti et al. (2008) were followed.

The original assay described by Astwood et al. (1996) recommends performing the digestion at pH 1.2. However, the FAO/WHO (2001) recommends using two pH conditions (pH 1.2 and pH 2.0). Two independent assays were conducted at each pH in this study. The assay was performed at 37 °C and samples removed at specific times and the activity of pepsin quenched by neutralization with carbonate buffer and Laemmli loading buffer. Samples were then heated to more than 70 °C for 5 min and frozen until analyzed by SDS–PAGE. The timed digestion samples were separated by SDS–PAGE (Novex 10–20% tris–glycine gels following the manufacturer's instructions – Life Technologies) fixed, and stained with coomassie or colloidal blue to evaluate the extent of digestion. All samples on each gel were from a single digestion experiment. The samples were heat to 95 °C, cooled and 10 µL each were loaded per well (1.47 µg protein per well). Bio-Rad Precision Plus molecular weight marker proteins were loaded in one outer well, Invitrogen Sharp Standards marker LC5800 was loaded in the opposite outer well. Stained gels were visualized in a Kodak Gel Logic 440 Image Station under white light transillumination.

Based on the purity of apoaequorin by densitometry of SDS PAGE, the limit of detection was estimated using serial dilutions of samples of purified test protein. Undigested samples representing 100% and 10% of the undigested protein were then included in the analytical digestion gels as reference standards.

3. Results and discussion

3.1. Verification of expression genetics

Verification of the integration of the expression construct into the host chromosome was determined by PCR and DNA sequencing of the amplified gene. The translated amino acid sequence was identical to the full-length, naturally occurring protein found in the jellyfish.

Following fermentation, the protein accumulated in the medium of the broth and samples from time points during fermentation were used for protein activity assays (Figure 1) and SDS PAGE analysis (Figure 2). Protein accumulation and luminescent activity agreed well for each time point. The SDS–PAGE demonstrated complete N-terminal cleavage of the signal peptide sequence, successful secretion of the protein into the medium, and confirmed the correct molecular weight of 22.3 kDa. The luminescent activity of the protein increased in accordance with the accumulation of the band density on the SDS–PAGE indicating production and secretion of a properly folded and fully functional protein into the medium. Apoaequorin was purified to homogeneity and concentrated to 20 mg/mL.

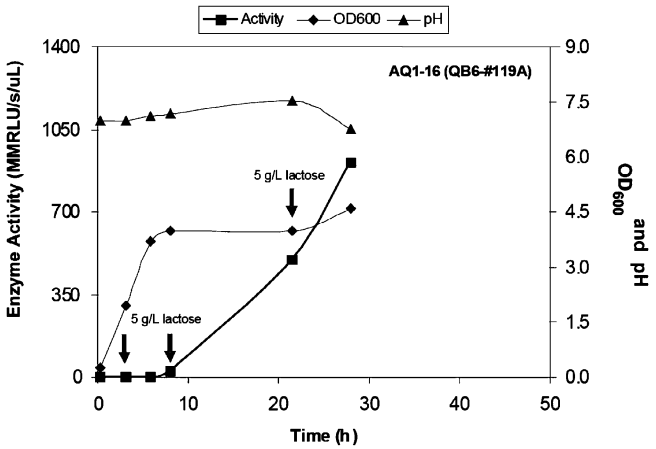

Figure 1. Kinetic results of the fermentation of the QB6 clone showing luminous activity of the charged apoaequorin (aequorin) as it increased as protein accumulated in the broth of the medium. Arrows indicate the time points for lactose induction of the gene. MMRLU/s/µL = maximum relative luminous units in millions of photons per second per µL of sample.

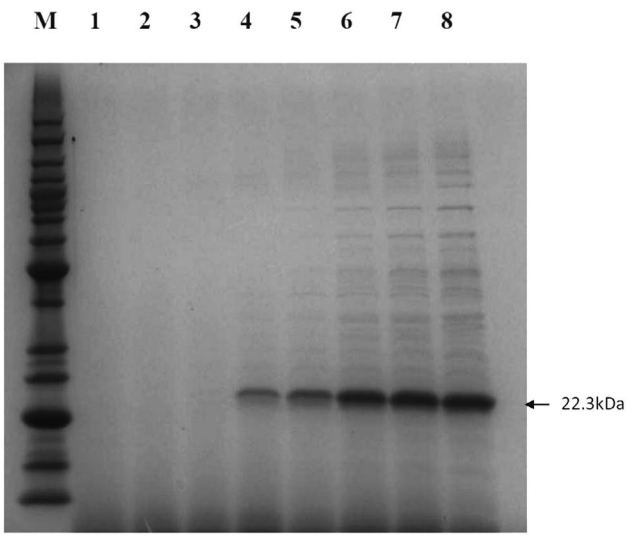

Figure 2. SDS PAGE and coomassie blue staining of samples from selected fermentation time points. Lane M, protein marker, Lane 1 broth before inoculation, Lane 2 at inoculation, Lane 3 at 0.2 h fermentation, Lane 4 at 3 h, Lane 5 at 6 h, Lane 6 at 8 h, Lane 7 at 22 h.

The first 30 residues of the protein were determined to be correct through N-terminal protein sequence analysis (Figure 3). This is as expected from the predicted DNA sequence of the expression cassette. The protein was produced and processed correctly, creating the same protein as found endogenously in the jellyfish.

3.2. In silico allergen sequence alignments

The full-length sequence of apoaequorin (196 amino acids), (UniProtKB/Swiss-Prot: P02592.1) was used to search the AllergenOnline.org database. No native signal peptide has been identified for this protein from *A. victoria*, according to Hamamoto et al. (2003) as a direct NCBI entry (2003). Results of the FASTA3 search of the apoaequorin against the AllergenOnline.org (version 10 and version 13) did not identify any significant alignment with any allergen. Scoring results for the four best scoring alignments demonstrate no significant matches with any allergen (Table 1). The highest scoring alignments were to a calcium-binding protein of olive pollen (*Olea europaea*) and a number of fish parvalbumin calcium-binding proteins. While the *E*-scores and identities suggest possible evolutionary homology, the alignments represent less than one-third of the length of apoaequorin and the similarities are not sufficiently high to suspect shared antibody binding cross-reactivity.

1	11	21	31
MTSKQYSVKL	TSDFDNPRWI	GRHKHMFNFL	DVNHNGKISL
41	51	61	71
DEMVYKASDI	VINNLGATPE	QAKRHKDAVE	AFFGGAGMKY
81	91	101	111
GVETDWPAYI	EGWKKLATDE	LEKYAKNEPT	LIRIWGDALF
121	131	141	151
DIVDKDQNGA	ITLDEWKAYT	KAAGIIQSSE	DCEETFRVCD
161	171	181	191
IDESGQLDVD	EMTRQHLGFW	YTMDPACEKL	YGGAVP

Figure 3. Amino acid sequence of apoaequorin (P02592). The encoded protein is 196 amino acids in length based on translation of the DNA sequence. The first 30 amino acids (underlined) of the expressed and purified protein were confirmed by LC–MSMS as described in the text.

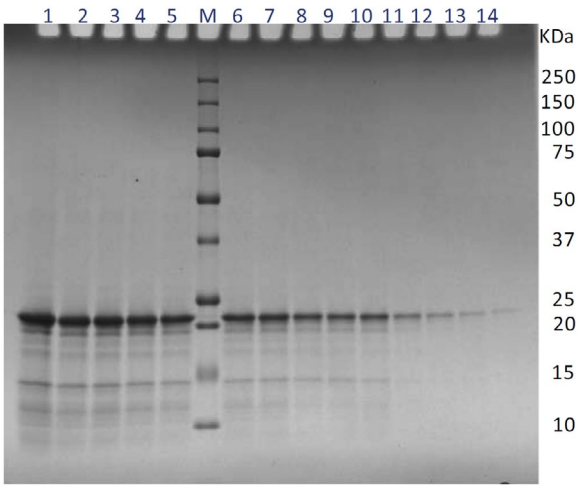

Figure 4. Colloidal blue stained SDS–PAGE gel apoaequorin for limit of detection. Samples of a dilution series of the apoaequorin was loaded and separated by SDS–PAGE using a 10 → 20% polyacrylamide tris–glycine gradient gel. A Bio-Rad Precision Plus marker protein was used to estimate MW. Proteins were detected by staining with colloidal brilliant blue G stain.

Lane	Description	Quantity of protein loaded/well
1	Apoaequorin (300%)	4.41 μg
2	Apoaequorin (200%)	2.94 μg
3	Apoaequorin (175%)	2.57 μg
4	Apoaequorin (150%)	2.21 μg
5	Apoaequorin (125%)	1.84 μg
M	Bio-Rad Precision Plus MW Marker	na
6	Apoaequorin (100%)	1.47 μg
7	Apoaequorin (80%)	1.18 μg
8	Apoaequorin (60%)	0.88 μg
9	Apoaequorin (50%)	0.74 μg
10	Apoaequorin (40%)	0.59 μg
11	Apoaequorin (20%)	0.29 μg
12	Apoaequorin (10%)	0.15 μg
13	Apoaequorin (5%)	0.07 μg
14	Apoaequorin (2.5%)	0.04 μg

The identity results from FASTA3 alignments using the sliding window of 80-amino acid algorithm did not identify any alignments of >35% identity to any entry in the AllergenOnline.org database (version 10 or 13). Furthermore, there were no identity matches of eight-contiguous amino acids between apoaequorin sequence and any entry in the AllergenOnline.org database.

The full-length apoaequorin was also compared to sequences in NCBI-Entrez using a keyword limit of "allerg*" on 20 June 2010 and again on 5 December 2013 (Table 2). No alignments were identified with *E* scores below "1 × e 10^{-7}", or with identity matches of greater than 35% over any alignment of more than 80 amino acids. These bioinformatics results did not identify any significant alignments to any known allergens. In contrast, when no keyword limit was used, many high-scoring identity matches were found to other photo-proteins as homologs of apoaequorin from other jellyfish relatives.

A PubMed search was conducted to identify any publications that suggest the species (*A. victoria*) or other members of related taxa are known to be significant inducers of allergy. Keyword search terms included "Aequorea AND allergen/allergy", "Aequoreidae AND allergen/allergy" (family) and "Cnidaria AND allergen/allergy" (phylum). The search was conducted on 19 June 2010. The only two publications identified by species search reports a study of protein

Table 1. Overall FASTA3 search of AllergenOnline version 10.0 with APQ. Four highest scoring alignments of known and putative allergens in AllergenOnline are shown to compare apoaequorin with version 10 of the AllergenOnline.org database using FASTA3.

Sequence GI#	Organism	Description	Length	*E* score	% Identity	aa alignment
3337403	*Olea europaea* olive	Ca^{+2} binding protein	84	0.088	33.3	51
131112	*Gadus callarias* codfish	Parvalbumin	113	0.24	35.7	56
77799800	*Trachurus japonicus* Japanese mackerel	Parvalbumin	107	0.37	34.5	55
14531018	*Theragra chalcogramma* Alaska pollock	Parvalbumin	109	0.68	32.7	55

Table 2. BLASTP of NCBI Entrez "allerg*" with APQ. The scoring alignments of ten best scoring matches with *E* scores below 10, to putative allergens in the NCBI Entrez database on 20 June, 2010, were compared to the full-length sequence of apoaequorin, using BLASTP.

Sequence GI#	Organism	Description	Length	*E* score	% Identity	aa alignment
3I7C_A	*Toxoplasma gondii* amoeba	Ca^{+2} dependent protein kinase	484	0.002	27%	90
3I79_A	*Toxoplasma gondii* amoeba	Ca^{+2} dependent protein kinase	484	0.003	27%	90
ABF18354.1	*Aedes aegypti* mosquito	Calmodulin	149	0.01	24%	153
XP_751821.2	*Aspergillus fumigates* fungus	Ca^{+2} dependent mitochondrial carrier protein	585	0.037	32%	59
BAB86193.1	*Oryza sativa* rice	Putative pollen allergen Jun 0 4	185	0.06	31%	73
AAX77686.1	*Ambroxia artemisiifoliashort* ragweed	Ca^{+2} bindin protein	160	0.069	24%	157
XP_751821.2	*Aspergillus fumigatus* fungi	Calmodulin	149	0.18	20%	158
O81092.1	*Olea europaea* rice	Polcalcin	84	0.22	33%	51
ADD19708.1	*Glossina morsitans* fly	Calmodulin	149	0.34	22%	150
AAT92161.1	*Ixodes pacificus* tick	Myosin light chain	174	0.39	22%	168

stability of heterologously expressed proteins using the Turnip Mosaic Virus (Chen et al., 2007) and Golgi trafficking of expressed proteins (Luo et al., 2006). Both use the green fluorescent protein of this species as a marker, with no indication the protein is an allergen. No publications were found using the family name "Aequoridae" with "allergen: or "allergy." Seventy-one publications were identified using the terms "Cnidaria AND allergy". Most of the 71 papers describe allergic reactions to contact with a variety of jellyfish, corals, and anemones or treatment of allergic reactions. Only one paper reported identification of the allergenic protein and that (Onizuka et al., 2001) was identified by search through the three publications identified by "Cnidaria AND allergen", describing allergy to an allergenic protein, of the coral *Dendronephthya nipponica*.

3.3. In vitro digestion assay

This study evaluated the stability of apoaequorin in two replicate assays using pepsin at both pH 1.2 and 2.0. A number of control steps were performed to ensure study validity including verifying that the activity of the pepsin at pH 1.2 and 2.0 was within the target activity range of 2750 units per mg of solid pepsin, plus or minus 23% on each day of assay. Preliminary SDS–PAGE results demonstrated that the apoaequorin was as expected in molecular weight and that the sensitivity of the stained gels and imaging system were sufficient to detect the protein from digestion samples below 10% of the starting material (Figure 4). Specific net intensity values from the Gel Logic 440 image station were plotted in a regression (Figure 5) with apparent good fit (r^2 = 0.91). Based on these data, the limit of detection was approximately 2.5% (0.04 µg) of apoaequorin 100% loading (1.47 µg) used in the digestion samples. This level of sensitivity was clearly sufficient to detect 10% residual protein in the digest.

The certified activity of the lot of pepsin from Worthington Biochemical used in this study was 2750 units per mg of solid. The activity was also verified in the laboratory and was within our quality control range. Representative stained gels of replicated digestion experiments at pH 1.2 and pH 2.0 demonstrated that apoaequorin is stable in acid alone (pH 1.2 or 2) for 60 min (lane 3). It was however, digested by 0.5 min in pepsin at acidic pH (lane 5) to the extent that it was less intense than the 10% apoaequorin control in quenched pepsin (Figure 6). Thus, the estimated time for 90% digestion was less than 30 s in all digestion experiments. Apoaequorin is therefore considered a rapidly digested protein.

4. Discussion

None of the results from the bioinformatics searches with the apoaequorin sequence met the criteria that suggest the protein is an allergen or poses a risk of allergenic cross-reactivity or would require further testing to evaluate potential risks. In addition, the general bioinformatics search of NCBI without a keyword limit demonstrated the protein is highly conserved across a broad range of taxa within jellyfish with no obvious published indication of allergy associated with homologous proteins. Furthermore, searches of published literature failed to identify a report, demonstrating allergy to *A. victoria* or other family members, but did identify one publication regarding a single protein in a different group within the phylum Cnidaria. A number of publications reported allergic or allergic-like reactions to various jellyfish, corals, and anemones in this phylum, but a paucity of information regarding the proteins implicated in sensitization. These results suggest that the entire phylum is not a significant cause of allergic reactions in humans.

The proteins that had regions of limited homology that did arise in the sequence search of the NCBI protein database were calcium-binding proteins and the alignments were in the region of calcium binding. This is not surprising since the signature motif for calcium-binding proteins is the EF-hand conformation that is created by the protein's helix-loop-helix secondary structure. However, it is not the primary sequence of the motif that is conserved but similar residues (size and charge) that is characteristic of calcium-binding proteins.

Epitope mapping of peptide fragments of calcium-binding proteins of known allergenicity has implicated some residues of an EF-hand motif as in the case of parvalbumin; however, the role of these residues in the tertiary conformation of the motif and dependence on the presence or absence of bound calcium ion (Perez-Gordo et al., 2013) makes extrapolation to globular proteins speculative. In any case, poor sequence alignment of the apoaequorin EF-hand domains with the calcium-binding domains of other proteins does not support the allergenic potential for

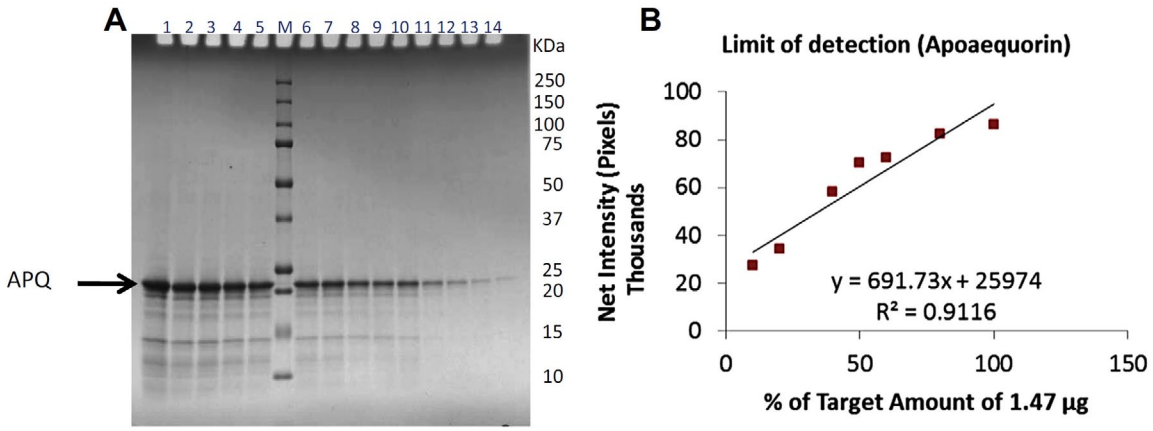

Figure 5. Densitometer readings for the limit of detection of apoaequorin from the colloidal blue stained SDS–PAGE gel (see Figure 1). The Kodak Gel Logic 440 with 1D software was used to make the estimation of stainable protein band. Excel was used to calculate the regression and equivalent protein content based on 100% being equal to 1.47 µg per lane. Target apoaequorin amount per lane is 1.47 µg. (A) Lanes 1–14 represent 30%, 200%, 175%, 150%, 125%, 100%, 80%, 60%, 50%, 40%, 20%, 10%, 5%, 2.5% of the target apoaequorin amount, respectively. (B) Densitometer readings for the limit of detection of apoaequorin from the colloidal blue stained SDS–PAGE gel (see sub-Figure A). The Kodak Gel Logic 440 with 1D software was used to make the estimation of stainable protein band. Excel was used to calculate the regression and equivalent protein content based on 100% being equal to 1.47 µg per lane.

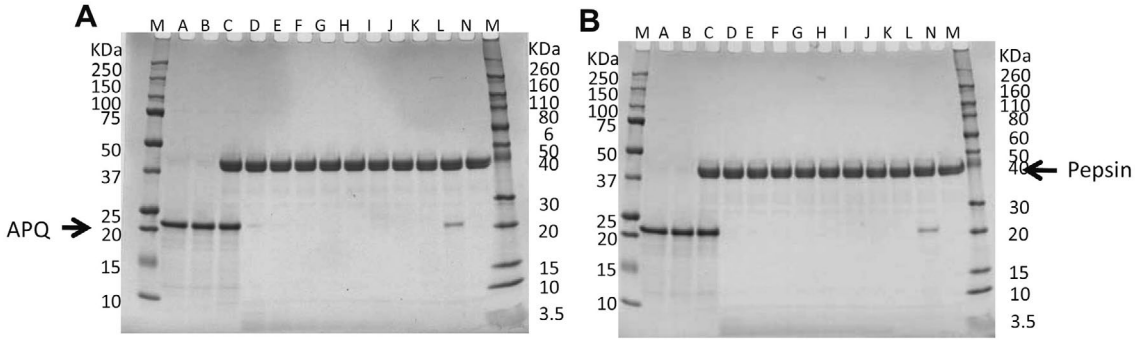

Figure 6. Detection of apoaequorin at pH 1.2 (sub-figure A) and pH 2.0 (sub-figure B) with 10,000 pepsin activity units per mg apoaequorin. Digestion products were separated in SDS–PAGE 10–20% gels under reducing conditions. Sample lanes: (m), BioRad# 161-0374 and Invitrogen LC5800 marker proteins; 1.47 mg of undigested apoaequorin without pepsin (A) at time 0; (B) at time 60 min; (C–J) apoaequorin plus pepsin at times 0, 0.5, 2, 5, 10, 20, 30, and 60 min of digestion (respectively); K, pepsin alone at time 0; N, pepsin alone at time 60 min; L, undigested apoaequorin 0.147 µg (10%) at time 0.

Lane	Description	Incubation time
M	Molecular weight marker Bio-Rad	na
A	Apoaequorin control without pepsin (P0)	0 min
B	Apoaequorin control without pepsin (P60)	60 min
C	Apoaequorin in SGF, (DC)	0 min
D	Apoaequorin in SGF, (D0.5)	0.5 min
E	Apoaequorin in SGF, (D2)	2 min
F	Apoaequorin in SGF, (D5)	5 min
G	Apoaequorin in SGF, (D10)	10 min
H	Apoaequorin in SGF, (D20)	20 min
I	Apoaequorin in SGF, (D30)	30 min
J	Apoaequorin in SGF, (D60)	60 min
K	Experimental control pepsin (E0)	0 min
L	Experimental control pepsin (E60)	60 min
N	10% apoaequorin with quenched pepsin (dC)	0 min
M	Molecular weight marker Invitrogen	na

the protein. Additionally, the ability to predict allergenic epitopes based on predicted three-dimensional modeling or other structural prediction tools has not been demonstrated through validation to be highly predicted and is thus not recognized as a risk assessment tool for food safety (Goodman and Tetteh, 2011). Instead primary sequence comparisons using FASTA (Pearson, 2000) or BLASTP (Pearson, 1995) have been demonstrated to be useful for identifying proteins that are sufficiently similar in primary structure to suspect possible shared IgE binding that might be clinically relevant for allergic consumers (Goodman et al., 2008). Thus, the criteria of the Codex Alimentarius Commission (2003) were followed in the bioinformatics analysis used to evaluate potential cross-reactivity. The results did not identify any significant matches to any allergenic protein. This demonstrates that apoaequorin is not expected to have any significant risk of allergic cross-reactivity for those who are allergic to known allergens. In fact, based on these

results, it would not be possible to identify allergic individuals who would be at a heightened risk by exposure to this protein.

In vitro digestion models are used widely to assess the digestibility of ingested substances. Resistance to digestion is typical of allergenic proteins. Based on this study, the rapid digestion characteristics of apoaequorin were similar to those of common non-allergenic dietary proteins.

From these data, there is no added concern of safety due to gastrointestinal stability of the protein.

Disclosures — Dr. R. Goodman reports grants from Quincy Bioscience LLC, during the conduct of the study. Dr. M.Y. Underwood has a patent US No. 7,671,015 issued, a patent US Appl. 12/694,427 pending, and a patent US Appl. 12/672,463 pending. Dr. A. Tetteh has nothing to disclose.

Appendix A. Supplementary data

ICMJE Disclosure forms for each of the authors are attached to the repository html cover page in a .zip file.

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Figure S1.