GR

ORIGINAL SUBMISSION

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September 2, 2014

GRN 000568

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OFFICE OF
FOOD ADDITIVE SAFETY

Office of Food Additive Safety (HFS-255)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Re: Generally Recognized As Safe ("GRAS") Notification for Apoaequorin

Dear Sir/Madam:

Pursuant to proposed 21 CFR 170.36 (62 FR 18960; April 17, 1997), our client, Quincy Bioscience Manufacturing, Inc. ("Quincy"), through Amin Talati, LLC as its attorneys, hereby provides notice of a claim that the food ingredient apoaequorin described in the enclosed notification document is exempt from the premarket approval requirement of the Federal Food, Drug, and Cosmetic Act because it has been determined to be Generally Recognized As Safe (GRAS), based on scientific procedures. As required, please find enclosed three copies of the notification.

On June 25, 2013, Quincy completed a Pre-GRAS Notification meeting with FDA's Office of Food Additive Safety (OFAS). During the meeting, FDA's OFAS provided Quincy with several recommendations regarding its proposed notification. Please note that FDA's recommendations and comments have been addressed in the enclosed notification. Additionally, Quincy retained two physicians to complete a comprehensive review of adverse events compiled for its dietary supplement Prevagen®, as well as to serve as advisers to the GRAS panel.[1] Reports from both physicians are also included with the enclosed notification.

Please also note that in December of 2013, FDA's Dietary Supplements Regulations Implementation Team of the Office of Nutrition, Labeling and Dietary Supplements issued a Certificate of Free Sale to Quincy for its Prevagen® dietary supplement, which contains the apoaequorin ingredient (*See* **Exhibit 1**).

[1] The Dietary Supplement and Nonprescription Drug Consumer Protection Act requires mandatory reporting to FDA of serious adverse events. However, by virtue of the statute itself adverse event reports are not causal in nature: *"The submission of any adverse event report in compliance with this section shall not be construed as an admission that the dietary supplement involved caused or contributed to the adverse event* (21 USC 379aa-1(g))."

Amin Talati, LLC · 55 West Monroe Street, Suite 3400 · Chicago, IL 60603-5022 · Office (312) 466-1033 · Fax (312) 884-7352 · www.AminTalati.com

If you have any questions or require additional information, please feel free to contact me by phone at 312.327.3381 or by email at Ashish@AminTalati.com.

Sincerely,

(b) (6)

Ashish Talati, J.D., M.S., RAC

Encl: GRAS Notification for Apoaequorin (3 copies)

Exhibit 1

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
College Park, MD 20740

December 27, 2013

Todd Olson
Quincy Bioscience
301 S Westfield Rd Ste 200
Madison, WI 53717

Dear Todd Olson:

This is in response to your letter dated November 21, 2013 addressed to the Food and Drug Administration requesting Certificates of Free Sale for products to be exported by Quincy Bioscience, 301 S Westfield Rd Ste 200, Madison, WI, 53717.

As requested, we are enclosing Certificates and "To Whom It May Concern" letters for the following product(s):

Prevagen (30 capsule), Prevagen Extra Strength (30 capsule), and Prevagen Professional (30 capsule)

Please be advised that the issuance of these certificates is intended solely to facilitate the export of the listed foods. They may not be used to promote the sale of the indicated products in the United States and do not suggest or imply that the Food and Drug Administration approves or sanctions the products or labels; or that the products or labels are in compliance with the Federal Food, Drug, and Cosmetic Act and regulations established there under; or that these certificates preclude the Agency from talking regulatory action against the products in the future, if warranted. It is the firm's responsibility to ensure that all foods and their labels comply with all applicable laws and regulations.

This certificate only attests to the status of your product under the Federal Food, Drug, and Cosmetic Act. You are responsible for assuring that your product, when exported to these countries is in compliance with all other applicable U.S. statutes and regulations.

The fee for providing this service is $10 for each certificate. Please submit a check or money order with the enclosed invoice payable to the Food and Drug Administration at the following address:

Department of Health and Human Services
Food and Drug Administration
DFM, Accounting Branch (HFA-120)
1350 Piccard Dr., Suite 200A
Rockville, Maryland 20850

Sincerely yours,

(b) (6)

Steven Casper, Ph.D.
Dietary Supplements Regulations Implementation Team
Division of Dietary Supplement Programs
Office of Nutrition, Labeling and Dietary Supplements
Center for Food Safety and Applied Nutrition

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
College Park, MD 20740

December 27, 2013

TO WHOM IT MAY CONCERN

We have reviewed correspondence on behalf of:

Quincy Bioscience
301 S Westfield Rd Ste 200
Madison, WI 53717

concerning the status of:

Prevagen (30 capsule), Prevagen Extra Strength (30 capsule), and Prevagen Professional (30 capsule)

These products are regulated by the Food and Drug Administration (FDA) pursuant to the requirements of the Federal Food, Drug, and Cosmetic Act (FD&C Act) and the Fair Packaging and Labeling Act (FPLA) and other related laws.

The Food and Drug Administration does not have statutory authority to approve any food or any food manufacturer or distributor of such products.

The above referenced products are under the jurisdiction of the Food and Drug Administration which has primary responsibility for the administration and enforcement of the FD&C Act and the FPLA and other related laws. We have not examined the specific products being offered for export or reviewed the labels. Under the FD&C Act, such products may be exported if:

1. It is not adulterated or misbranded and it meets the other requirements of the FD&C Act for marketing in the U.S.; or

2. It cannot be lawfully marketed in the U.S. but meets the requirements of section 801(e) of the FD&C Act (21 U.S.C. 381(e)).

Sincerely yours,

(b) (6)

Steven Casper, Ph.D.
Dietary Supplements Regulations Implementation Team
Division of Dietary Supplement Programs
Office of Nutrition, Labeling and Dietary Supplements
Center for Food Safety and Applied Nutrition

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
College Park, MD 20740

CERTIFICATE OF FREE SALE

1. Pursuant to the provisions of Rule 44 of the Federal Rules of Civil Procedure, I hereby certify that the copy attached (see attached list) is a true copy of material on file in the Food and Drug Administration, Department of Health and Human Services and is a part of the official records of said Administration and Department.

Letter Dated:

December 27, 2013

To Whom it May Concern

from, Steven Casper

regarding

Prevagen (30 capsule)
Prevagen Extra Strength (30 capsule)
Prevagen Professional (30 capsule)

2. In witness whereof, I have pursuant to the provisions of Title 42, United States Code, Section 3505, and 1410.20 of the FDA Staff Manual Guide, hereto set my hand and cause the seal of the Department of Health and Human Services to be affixed this 27th day of December, 2013.

(b) (6)

Daniel Fabricant, Ph.D.
Director, Division of Dietary and Supplement Programs
Office of Nutrition, Labeling and Dietary Supplements
Center for Food Safety and Applied Nutrition

By direction of the Secretary of Health and Human Services

This Certificate expires on December 27, 2015.

GRAS NOTIFICATION

I. Claim of GRAS Status

A. Claim of Exemption from the Requirement for Premarket Approval Requirements Pursuant to Proposed 21 CFR § 170.36(c)(1)

Quincy Bioscience Manufacturing, Inc. (the notifier) has determined that Apoaequorin is Generally Recognized As Safe, consistent with Section 201(s) of the *Federal Food, Drug, and Cosmetic Act*. This determination is based on scientific procedures as described in the following sections, under the conditions of its intended use as a food ingredient. Therefore, the use of apoaequorin is exempt from the requirement of premarket approval.

Signed,

(b) (6) [redacted]

Date 9/2/2014

Ashish Talati, J.D., M.S., RAC

Attorney for:

Quincy Bioscience Manufacturing, Inc.
301 S Westfield Rd, Suite 200
Madison, WI 53717
USA

B. Name and Address of Notifier:

Mark Y. Underwood
Quincy Bioscience Manufacturing, Inc.
301 S Westfield Rd, Suite 200
Madison, WI 53717
USA
Tel: +1-608-827-8000

through its attorney,

Ashish Talati, J.D., M.S., RAC
Amin Talati, LLC
55 West Monroe Street, Suite 3400
Chicago, IL 60603
Tel: 312-327-3381
Fax: 312-884-7352
Email: Ashish@AminTalati.com

C. Common or Usual Name of the Notified Substance:

The common name of the substance of this notification is Apoaequorin.

D. Conditions of Intended Use in Food

Apoaequorin is intended for use as a food ingredient at levels up to 6 mg of Apoaequorin *per* serving (reference amounts customarily consumed, 21 CFR § 101.12) in dairy products such as milk shake and smoothies when not precluded by a standard of identity. The intended use of Apoaequorin in above mentioned food categories is estimated to result in mean and maximum (90th percentile) intake of 8.94 and 19.05 mg/person/day, respectively. For an individual weighing 60 kg the resulting intake will be 0.33 mg/kg body weight/day.

Apoaequorin is not proposed for uses in foods that are intended for infants and toddlers, such as infant formulas or foods formulated for babies or toddlers, as well as it is not intended for use in meat and poultry products that come under USDA jurisdictions.

E. Basis for GRAS Determination:

In accordance with 21 CFR 170.30, the intended use of Apoaequorin has been determined to be Generally Recognized As Safe (GRAS) based on scientific procedures. The GRAS determination is supported by the opinion of the Expert Panel. A comprehensive search of the scientific literature was also utilized for this determination. There exists sufficient qualitative and quantitative scientific evidence, including human and animal data to determine safety-in-use for Apoaequorin. The safety determination of Apoaequorin is based on the totality of available evidence. Apoaequorin produced by Quincy Bioscience is identical to the naturally occurring protein present in jellyfish. The safety data on Apoaequorin protein preparation includes subchronic toxicity studies in rats, *in vitro* genotoxicity study, lack of alleginicity potentials, *in vitro* enzymatic hydrolysis (metabolism),

and human tolerance following oral ingestion in a clinical trial. On the basis of scientific procedures[1], Quincy Bioscience Manufacturing, Inc. (Quincy) considers the consumption of Apoaequorin, as a food ingredient to be safe at levels up to 19.05 mg/person/day (0.33 mg/kg bw/day).

F. Availability of Information:

The data and information that forms the basis for this GRAS determination will be provided to Food and Drug Administration upon request. The primary toxicologist, Dr. Madhusudan G. Soni, responsible for the preparation of this GRAS monograph and who is also a member of the expert panel can be contacted for the data and information that forms the basis for this GRAS determination. The data and information will be available for FDA review and copying at reasonable times at the offices of:

Madhusudan G. Soni, PhD, FACN, FATS
Soni & Associates Inc.
749 46th Square
Vero Beach, FL 32068

Telephone: +1- 772-299-0746;

Email: msoni@soniassociates.net or sonim@bellsouth.net

II. Detailed Information About the Identity of the Notified Substance:

A. Chemical name:

Apoaequorin

B. Chemical Abstract Registry Number:

845410-67-5

C. Physical Characteristics

Light tan, dry powder without any characteristic taste and odor

D. Typical Composition and Specifications

Food grade specifications of Apoaequorin are presented in Table II-D.1. Analytical data from five non-consecutive manufacturing lots is presented in Appendix I.

[1] 21 CFR §170.3 Definitions. (h) Scientific procedures include those human, animal, analytical, and other scientific studies, whether published or unpublished, appropriate to establish the safety of a substance.

Table II.D.1. Standard Specifications of Apoaequorin Preparation*

Parameter	Standard specifications	Assay method
Description	Light tan color powder	Visual
Apoaequorin content	50-60%	RP-HPLC
Identification	**A. HPLC**: peak must be within 0.5 min of average std retention time. **B. SDS PAGE**: molecular weight must align with standard on gel.	HPLC/SDS PAGE
Moisture	< 7%	AOAC 927.05
pH	5.5 - 6.5	AOAC 943-02
Residue on ignition	25-35%	USP <281>
Solubility (water)	70 mg/mL	In House
Heavy metals		
Total heavy metals	< 5 ppm	USP <231>
Arsenic	< 1 ppm	AOAC 993.14
Cadmium	< 1 ppm	AOAC 993.14
Lead	< 1 ppm	AOAC 993.14
Mercury	< 1 ppm	AOAC 993.14
Microbiological assays		
Aerobic plate count	<100,000 CFU/g	BAM 8th Edition
Coliform counts	<10 CFU/g	BAM 8th Edition
Escherichia coli	<10 CFU/g	BAM 8th Edition
Staphylococcus aureus	<10 CFU/g	BAM 8th Edition
Mold counts	<1000 CFU/g	BAM 8th Edition
Yeast count	<1000 CFU/g	BAM 8th Edition
Salmonella	Negative	BAM 8th Edition

E. Manufacturing process

Apoaequorin protein preparation is manufactured according to current good manufacturing practices (cGMP), as presented in Figure E.1. Although this protein occurs at very low levels in jellyfish, Apoaequorin protein, the subject of present GRAS is produced through recombinant fermentation of innocuous *Escherichia coli* strain BL21 (DE3) QB69A, a non-toxigenic and non-pathogenic microorganism. The production process is fully characterized and standardized. The DNA introduced into the *E. coli* BL21 (DE3) recipient strain is well characterized and shown to be safe. The protein is recovered from the fermentation broth by filtration, purification and drying. The fermentation medium has been optimized for production of the Apoaequorin protein. The raw materials used in the manufacturing are food grade and meet USP or multicompendial standards. The ion exchange resins used in the manufacturing are in compliance with ISO 9001 and are registered with the FDA as a raw material acceptable for use in the manufacturing of pharmaceutical proteins. The manufacturing methods of fermentation, filtration and purification by ion exchange as well as the choice and identity testing of raw materials is optimized to reduce the introduction of impurities to the final product and eliminate potential or theoretical metabolic contaminants from the dried ingredient.

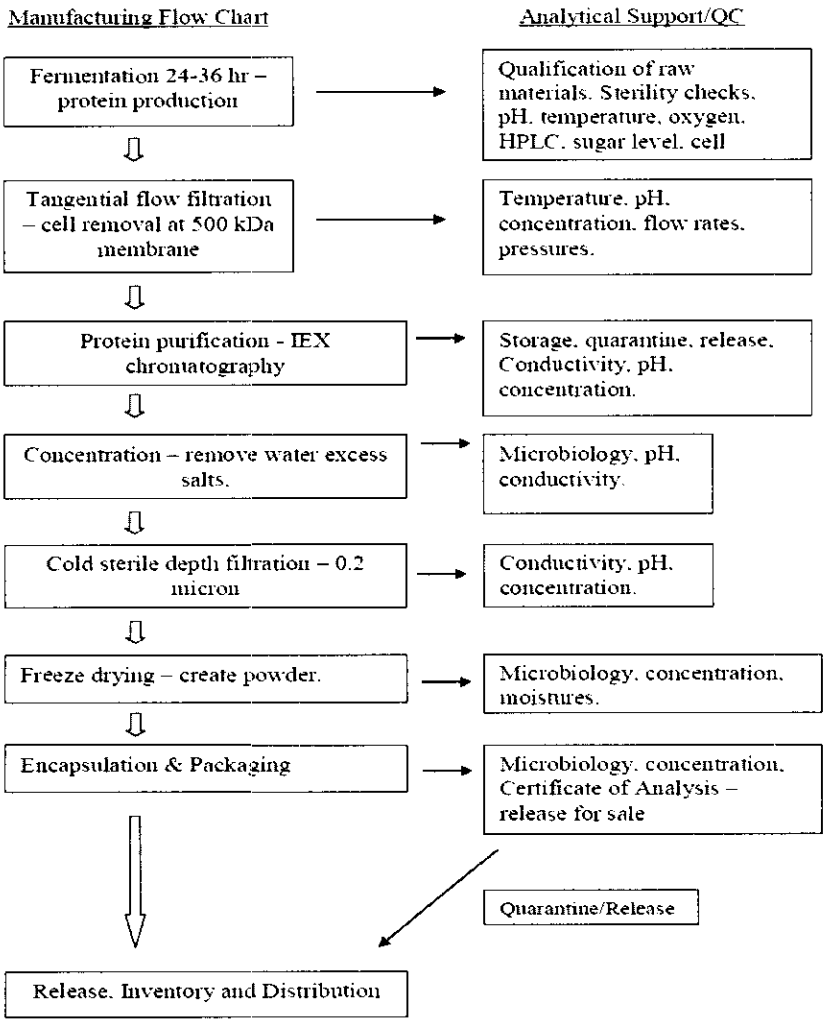

Figure E.1. Manufacturing process of Apoaequorin preparation (Quincy Bioscience, 2012)

III. Summary of the Basis for the Notifier's Determination that Apoaequorin is GRAS

The determination that apoaequorin is GRAS is based on scientific procedures. A comprehensive search of the scientific literature for safety and toxicity information on apoaequorin was conducted through June 2014 and was also utilized for this assessment. Based on a critical evaluation of the pertinent data and information summarized here and employing scientific procedures, it is determined that the addition of apoequorin to the selected foods described in this notice and at use levels of 6 mg/serving (in accordance with established reference amounts customarily consumed, 21 CFR 101.12) meeting the specification cited above and manufactured according to current Good Manufacturing Practice, is GRAS under the conditions of intended use as specified herein.

In coming to this decision that apoaequorin is GRAS, Quincy relied upon the conclusion that apoaequorin did not pose any toxicological hazards or safety concerns at the intended use

levels, as well as on published toxicology studies and other articles relating to the safety of the product. Other qualified and competent scientists, reviewing the same publicly available toxicological and safety information, would reach the same conclusion.

IV. Basis for a Conclusion that Apoaequorin is GRAS for its Intended Use.

An independent panel of recognized experts, qualified by their scientific training and relevant national and international experience to evaluate the safety of food and food ingredients, was convened to determine the safety of apoaequorin. The Expert Panel consisted of the following individuals: Professor John Thomas, Ph.D., FATS (Indiana University School of Medicine); Robert L. Martin, Ph.D. (Retired FDA Deputy Director); Professor Michael W. Pariza, Ph.D. (University of Wisconsin-Madison). In coming to the conclusion Apoaequorin is safe, the Expert Panelists also used following advisers: Professor Gary M. Williams, M.D. (New York Medical College); Professor Xavier Pi-Sunyer, M.D. (Columbia University College of Physicians and Surgeons), and Madhusudan G. Soni, PhD (Food Ingredient Safety Consultant). Based on a critical evaluation of the pertinent data and information summarized herein, the Expert Panel members have individually and collectively determined by scientific procedures that the addition of apoaequorin as a food ingredient at levels up to 6 mg of Apoaequorin *per* serving (reference amounts customarily consumed, 21 CFR § 101.12) in milk shake and smoothies when not precluded by a standard of identity, resulting in a mean and 90th percentile estimated daily intake of 8.94 and 19.05 mg Apoaequorin/person/day, respectively is GRAS. It is also their opinion that other qualified and competent scientists, reviewing the same publicly available toxicological and safety information, would reach the same conclusion (see enclosed Expert Panel Statement).

EXPERT PANEL STATEMENT

DETERMINATION OF THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF APOAEQUORIN PROTEIN PREPARATION

Prepared for
Quincy Bioscience
301 S. Westfield Road, Suite 200
Madison, WI 53717

&

Amin Talati, LLC,
55 W. Monroe St, Suite 3400
Chicago, IL 60603

Prepared by
Soni & Associates Inc.
749 46th Square
Vero Beach, FL 32968, USA

Panel Members

John A. Thomas, PhD, FACT, DATS
Robert L. Martin, PhD
Michael W. Pariza, PhD
Madhusudan G. Soni, PhD, FACN, FATS
Prof. Gary M. Williams, M.D., DABT, FRCPath
Xavier Pi-Sunyer, MD, MPH

August, 2014

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EXPERT PANEL STATEMENT

DETERMINATION OF THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF APOAEQUORIN PROTEIN PREPARATION

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DETERMINATION OF THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF APOAEQUORIN PROTEIN PREPARATION

1. INTRODUCTION

The undersigned, an independent panel of recognized experts (hereinafter referred to as the Expert Panel)[1], qualified by their scientific training and relevant national and international experience to evaluate the safety of food and food ingredients, was convened by Soni & Associates Inc., at the request of Quincy Bioscience (Madison, WI) and Amin Talati, LLC (Chicago, IL) to determine the Generally Recognized As Safe (GRAS) status of Apoaequorin, a protein preparation for use as a food ingredient in milkshakes and smoothies at use levels of 6 mg/serving (reference amounts customarily consumed, 21 CFR § 101.12). A comprehensive search of the scientific literature for safety and toxicity information on Apoaequorin protein preparation was conducted through June 2014 and made available to the Expert Panel. The Expert Panel independently and critically evaluated materials submitted by Quincy Bioscience and other information deemed appropriate or necessary. Following an independent, critical evaluation, the Expert Panel conferred on August 13, 2014 and unanimously agreed to the decision described herein.

1.1. Background

Aequorin, a photoprotein is found in luminescent jellyfish (various *Aequorea* species, e.g., *Aequorea victoria*) and a variety of other marine organisms. This photoprotein is composed of two distinct units, the apoprotein, Apoaequorin and the prosthetic group coelenterazine, a luciferin (obtained from the animal's diet). Aequorin emits blue light by an intramolecular reaction when mixed with Ca^{2+} ions (Shimomura et al., 1974). For over four decades, the protein has been used as a bio-luminescent indicator to elucidate the role of calcium as a second messenger in cell signaling cascades (Kendall and Badminton, 1998; Vernon et al., 2002). The protein, Apoaequorin, has been used in multiple cell types and has been localized to important intracellular compartments. The protein consists of 196 amino acid residues and is composed of all common dietary amino acids making it a complete protein source.

The role of calcium in biology, including regulatory functions such as neuromuscular transmission, cellular secretions and blood clotting is widely recognized (Hendler and Rorvik, 2001). Alterations in calcium homeostasis have been claimed to underlie the reduced cellular function characteristic of the normal aging process of the brain (Foster et al., 1997, Kelly et al., 2006, Thibault et al., 2007). The endocrine system that helps maintain calcium homeostasis is integrated and complex. Calcium-binding proteins have been recognized as protective factors in neuronal populations susceptible to toxicity via calcium and calcium-mediated events (Iacopino et al., 1990; Rogers et al., 1990; Foster et al., 2001; Mattson et al., 2002; Kumar et al., 2009). The role of these proteins in the human body as protective agents have also been recognized (Heizmann and Braun, 1992). The available information indicate that calcium-channel blocking agents control the calcium ion from crossing calcium channels in order to reduce the signaling by

[1]Modeled after that described in section 201(s) of the Federal Food, Drug, and Cosmetic Act, As Amended. See also attachments (curriculum vitae) documenting the expertise of the Panel members.

the calcium ion and to reduce the adverse effects of calcium crossing through these channels. In *in vitro* studies, Apoaequorin has been used to measure calcium response to extracellular stimuli in yeast cells (Nakajima-Shimada et al., 1991) and cell death following an ischemic insult (Choi, 1992). In another study, Detert et al. (2009) reported that direct hippocampal infusion of Apoaequorin in rats prior to oxygen and glucose deprivation showed protection of neurons from the ischemic insult. The available clinical evidence indicates that daily intake of Apoaequorin for 3 months may support improvements in recall memory in otherwise healthy adults (Underwood et al., 2011). Given the potential use of Apoaequorin as a dietary supplement in possible support of cognitive health, Quincy Bioscience intends to market a standardized Apoaequorin protein preparation product, under the trade name Prevagen®, for use as a dietary ingredient in milkshakes and smoothies.

1.2. Description

Apoaequorin, a standardized protein preparation produced by microorganism recombinant technology, is a light tan colored powder. General descriptive parameters and properties of Apoaequorin protein preparation manufactured as Prevagen® by Quincy Bioscience are summarized in Table 1.

Table 1. General Descriptive Characteristics of Apoaequorin

Parameter	Description (Quincy Biosceince, 2012)*
Chemical name	Apoaequorin
CAS Number	845410-67-5
Physical characteristics	Solid, Dry Powder
Color	Light tan
Odor	None
Chemical composition	Made of 196 amino acids
Molecular weight	22.3 kDA
Solubility	Up to 70 mg/mL in water
Functional Use	Dietary supplement
Shelf life	12 months

*Based on information provided by Quincy Bioscience (2012)

1.3. Specifications and Identity

Food grade specifications of Apoaequorin protein preparation (Prevagen®) have been established by Quincy Bioscience and are presented in Table 2. As moisture can reduce protein stability, moisture content is tested on finished goods to assure it remains low. Microbiological analysis is performed for organisms typically screened for food borne pathogens either by AOAC or BAM methods. The Apoaequorin protein powder is standardized to contain approximately 50-60% apoaequorin protein, 40-50% sodium chloride, and minor components of the purification buffer such as acetic acid (<7%). Analytical results from five non-consecutive lots (Appendix I) demonstrate that Apoaequorin preparation is consistently manufactured to meet these specifications. Among the five lots, in one lot the Apoaequorin content was relatively high as a result of the purification process. The variation in the dried powder depends on the use of sodium chloride and the purification process. In recent production lots, Quincy Bioscience has tightened the variation of Apoaequorin to 50-60%. The stability results from five lots of

Apoaequorin (Appendix II) show that the product is stable for over 3 years. Apoaequorin, produced using recombinant technology is identical to the naturally found Apoaequorin protein in jellyfish.

Table 2. Standard Specifications of Apoaequorin Preparation*

Parameter	Standard specifications	Assay method
Description	Light tan color powder	Visual
Apoaequorin content	50-60%	RP-HPLC
Identification	**A. HPLC:** peak must be within 0.5 min of average std retention time. **B. SDS PAGE**: molecular weight must align with standard on gel.	HPLC/SDS PAGE
Moisture	< 7%	AOAC 927.05
pH	5.5 - 6.5	AOAC 943-02
Residue on ignition	25-35%	USP <281>
Solubility (water)	70 mg/mL	In House
Heavy metals		
Total heavy metals	< 5 ppm	USP <231>
Arsenic	< 1 ppm	AOAC 993.14
Cadmium	< 1 ppm	AOAC 993.14
Lead	< 1 ppm	AOAC 993.14
Mercury	< 1 ppm	AOAC 993.14
Microbiological assays		
Aerobic plate count	<100,000 CFU/g	BAM 8th Edition
Coliform counts	<10 CFU/g	BAM 8th Edition
Escherichia coli	<10 CFU/g	BAM 8th Edition
Staphylococcus aureus	<10 CFU/g	BAM 8th Edition
Mold counts	<1000 CFU/g	BAM 8th Edition
Yeast count	<1000 CFU/g	BAM 8th Edition
Salmonella	Negative	BAM 8th Edition

*Based on information provided by Quincy Bioscience (2012)

1.4. Chemistry

Apoaequorin is a protein composed of 196 amino acids. This protein is composed of all 20 amino acids making it a complete protein source. The number of individual amino acids along with frequency of occurrence in Apoaequorin is presented in Table 3. For a small protein of 196 amino acid residues, it is particularly rich in aromatic amino acids such as tryptophan, (3% of the total amino acid composition). Additionally, the protein is rich in several other aromatic amino acids such as tyrosine (4%), and phenylalanine (4%). The molecular weight of this protein is 22.3 kDA. The amino acid sequence for Apoaequorin is presented below and can be found in the Swiss-Protein database under the accession number P02592:

MTSKQYSVKLTSDFDNPRWIGRHKHMFNFLDVNHNGKISLDEMVYK
ASDIVINNLGATPEQAKRHKDAVEAFFGGAGMKYGVETDWPAYIEG
WKKLATDELEKYAKNEPTLIRIWGDALFDIVDKDQNGAITLDEWKA
YTKAAGIIQSSEDCEETFRVCDIDESGQLDVDEMTRQHLGFWYTMDP
ACEKLYGGAVP

Table 3. Chemical Composition of Apoaequorin*

Amino Acid	Count	Frequency	Amino Acid	Count	Frequency
Alanine (A)	16	0,082	Methionine (M)	6	0,031
Cysteine (C)	3	0,015	Asparagine (N)	8	0,041
Aspartic Acid (D)	19	0,097	Proline (P)	6	0,031
Glutamic Acid (E)	15	0,077	Glutamine (Q)	6	0,031
Phenylalanine (F)	8	0,041	Arginine (R)	6	0,031
Glycine (G)	15	0,077	Serine (S)	8	0,041
Histidine (H)	5	0,026	Threonine (T)	11	0,056
Isoleucine (I)	12	0,061	Valine (V)	10	0,051
Lysine (K)	16	0,082	Tryptophan (W)	6	0,031
Leucine (L)	12	0,061	Tyrosine (Y)	8	0,041

*Based on information provided by Quincy Bioscience (2012)

1.5. Manufacturing Process

1.5.1. Overall Process

Apoaequorin protein preparation is manufactured according to current good manufacturing practices (cGMP), as presented in Figure 1. As this protein occurs at very low levels in jellyfish, it is difficult to produce sufficient quantities of Apoaequorin from jellyfish or other natural sources. Accordingly, an improved means for producing Apoaequorin in sufficient quantities for commercial utilization is required. This was achieved by a proprietary process of fusing the Apoaequorin cDNA to a signal peptide coding sequence that targets the inner membrane of *Escherichia coli*; to the periplasmic space. The gene is under the control of the T-7 promoter; a sequence of DNA specific for T7 polymerase binding and under the control of the uv-lacZ T7 polymerase gene which was also integrated into the genome of *E. coli* by the commercial supplier of the strain, BL21 (DE3). Expression of cDNA in *E. coli* resulted in the production of large excess of the recombinant protein that is released into the culture medium. Thus, the Apoaequorin protein is produced through recombinant fermentation of innocuous *E. coli* strain BL21 (DE3) QB69A, a non-toxigenic and non-pathogenic microorganism. The protein is then recovered from the fermentation broth by filtration, purification and drying. The details of genetic modification of *E. coli* strain BL21 (Quincy Bioscience confidential information) are summarized below and also included in Appendix III.

The microbial strain, *E. coli* B21 (DE3) used in the production of Apoaequorin has been fully characterized for its safe use in the protein production, including characterization of the modifications that have been made to the host organism for Apoaequorin protein production. These characteristics demonstrate that the procedures used to modify the host are appropriate for food use. The *E. coli* BL21 (DE3) strain selected for Apoaequorin production has a history of safe use in producing recombinant proteins for uses in both academic laboratories and industrially for pharmaceutical manufacture. Its genome has been fully sequenced and compared against several other strains of *E. coli* to assist in verifying its lineage. Based on all this information, this strain is considered a Biosafety Level 1 microorganism. Quincy Bioscience

genetic modifications were performed according to standard methods with verifiable results (Hamilton et al., 1989). Additionally, Dr. Pariza independently reviewed the biology of *E. coli* BL21(DE3), relevant information available in the peer-reviewed scientific literature and information provided by Quincy Bioscience, and concluded that the *E. coli* BL21(DE3) production strain developed by Quincy Bioscience is appropriate for use in the manufacture of a food-grade ingredient (Opinion Letter- Attached Separately).

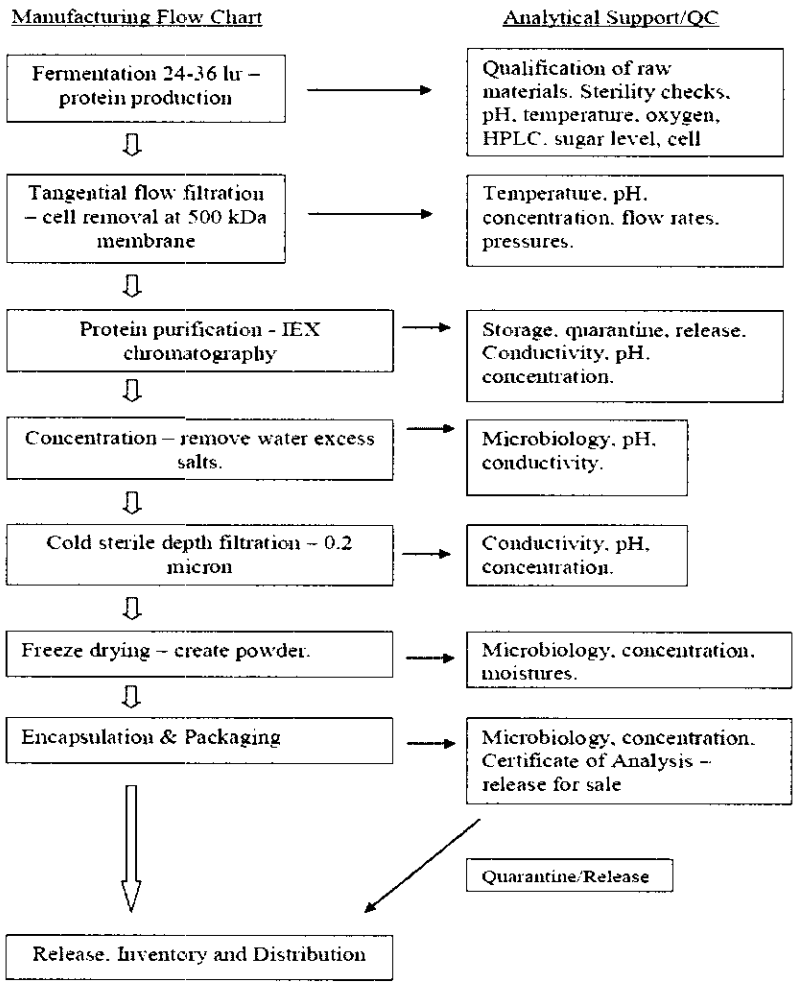

Figure 1. Manufacturing process of Apoaequorin preparation (Quincy Bioscience, 2012)

1.5.2. Host Strain Modification and Characterization

Using the host strain for the over-expression of the Apoaequorin protein, a further modification of the organism was required to assure that the gene of interest was placed into the host's genome for genetic stability of the transformation and to eliminate the use of certain chemicals, some of which are known allergens and irritants, from the manufacturing process. For this, a synthetic, codon-optimized version of the jellyfish gene was made and ligated to a plasmid vector. The construction resulted in a T7 promoter driven transcript with a secretion signal in

frame with the N-terminal methionine codon for the Apoaequorin gene. Subcloning of the expression cassette into a second vector for site-specific recombination into the host genome and subsequent screening of the clones assured that this protocol results in a insertion into a genome that did not disrupt the organism's genetic repertoire in such a way as to create novel proteins or metabolites which might create allergenic or other noxious entities in the manufacturing process. Furthermore, the chromosomal insertion does not need antibiotics to select for the stability of strain, some of which are known allergens, e.g., ampicillin. The entire protein expression system was designed to allow gene expression to be induced by the milk sugar lactose rather than the chemical isopropyl β-D-1-thiogalactopyranoside (IPTG), a synthetic analogue of allolactose. IPTG is a known irritant and not metabolized by the organism. Secretion of the Apoaequorin protein is mediated by the PelB leader sequence and the leader is cleaved by the host's signal secretion protease. This results in the complete 196 amino acid sequence being liberated into the cell's periplasmic space. The protein is released into the medium making cell removal and purification simpler.

Furthermore, a full characterization of these modifications was also accomplished to thoroughly develop a quality assessment and assure safe use of the strain at industrial levels. This included southern blot analysis for the presence of the transgene and the absence of vector DNA in the genome. Full details of the cloning and screening project are described in Appendix III.

A full characterization of the producing strain was performed and this included: Identification of the host organism; Southern blot analysis of the host genome demonstrating insertion of gene sequence and the absence of vector DNA; Purity test of the Master Cell Bank; Phage testing of the Master Cell Bank; Post storage viability testing of the Master Cell Bank; Verification of the inserted sequence by DNA sequence analysis of PCR; N-terminal sequencing of the expressed protein; Calcium-binding activity of the produced protein by luminometry; Confirmation of molecular weight by SDS PAGE; and, Confirmation of pI by isoelectric focusing. A working cell bank is made from the Master Cell bank and qualified by testing for purity, post freeze viability, protein production and is kept at the contract manufacturing organization for use in developing the seed strain to inoculate the production fermenter. All these procedures are intended to assure that a pure and productive strain with known nontoxic/nonpathogenic background is being used in the production of Apoaequorin.

1.5.3. Details of Fermentation Process

Fermentation and cell removal are performed by a large scale producer of biologics and probiotics that follows NIH guidelines for large-scale fermentation of recombinant organisms. Clarification of the fermentation mash through hollow fiber tangential flow filtration removes cells and results in a clear aqueous broth in which the protein is suspended. Purification of the cell free broth is performed using multiple batch-wise anion exchange column chromatography steps. The purified protein batches are combined and condensed by hollow fiber (Tangential Flow Filtration) to reduce volume further. The purified and condensed protein is then microfiltered to remove any microbial contaminants/impurities occurring throughout the purification process. The liquid form of the purified protein is then freeze dried and tested for potency and microbial contaminants. The product is vacuum-packed and then shipped for storage, under temperature controlled conditions. Final products are made by blending, encapsulation and packaging. Both in-process and release analytical analysis are performed at key points in the

process. All manufacturing sites are GMP compliant and have been audited by the FDA within the past 3 years.

The raw materials used in the manufacturing are food grade and meet USP or multicompendial standards. A list for raw materials used in seed flask, seed tank and production tank fermentation processes are identified in Table 4. The medium has been optimized for production of the Apoaequorin protein. Adjustments of pH are made with ammonia or phosphoric acid. Raw materials for the recovery process of the protein are identified in Table 5. The ion exchange resins used in the manufacturing are in compliance with ISO 9001 and are registered with the FDA as a raw material acceptable for use in the manufacturing of pharmaceutical proteins.

Table 4. Raw Materials Used in the Fermentation Process*

Material	Usage rate (g/L)
Purified Water (reverse osmosis)	
Hydrolyzed Casein	1-10
Yeast Extract	1-10
Ammonium Phosphate Dibasic	0 to 0.5
Sodium chloride	0-5
Soy flour digest	0-10
Magnesium Sulfate	0-1
Citric acid	0-2
Potassium Phosphate Monobasic	0 to 8.0
Glucose/Dextrose	0-10
Lactose	0-15
Antifoam-Industrial DF204-204kg	0.25
Trace minerals	Optional
Ferrous sulfate heptahydrate	0-0.025
Cupric Sulfate 5-Hydrate, fine Crystal	0-0.006
Manganous Sulfate Monohydrate, Powder	0-0.003
Sodium Borate 10-Hydrate, Crystal	0-0.001
Sodium Molybdate Dihydrate	0-0.001
Zinc Sulfate 7-Hydrate, Crystal	0-0.005
Calcium Chloride	0-0.0004

*Based on information provided by Quincy Bioscience (2012)

Table 5. Raw Material Used in Recovery Process*

Recovery and Purification Process	Concentration
Purified Water (reverse osmosis)	
Sodium chloride	25 mM to 1 M
TRIS Base Buffer	10-25 mM
EDTA	1-5 mM
Adjustments for pH are made with diluted caustic soda, hydrochloric acid, acetic acid or sulfuric acid.	
Chromatography resin Anion exchange such as DEAE FF Q Sepharose FF	

*Based on information provided by Quincy Bioscience (2012)

1.5.4. Process Impurities/External Contaminants

The choice of the host organism and the methods used to develop, integrate and verify gene construction and function have been fully characterized for a safe protein production system for the Apoaequorin protein to be used for human consumption. The strain of prokaryote [*E.* coli B21 (DE3)] selected for modification is a non-toxigenic and non-pathogenic organism, well characterized and typically used for the production of recombinant proteins for both analytical method and injectable therapeutic protein production (details on the strain selection and development described below). The choice of a secretion system permits the removal of host cells from the broth which contains the protein.

The manufacturing methods of fermentation, filtration and purification by ion exchange as well as the choice and identity testing of raw materials reduce the introduction of impurities to the final product and eliminate potential or theoretical metabolic contaminants from the dried ingredient. The use of 500 kDa pore size membrane filtration removes cells and eliminates the carryover of cellular lipids and DNA as well as other large macromolecules potentially released from the host. Column chromatography selectively captures the soluble protein in the clarified broth and permits spent medium components and un-metabolized ingredients to flow through the columns. The captured protein is then washed as it is bound to the column with a bacteriostatic buffer and then removed from off the resin by a salt gradient. This results in a solution of purified protein which is then labeled and refrigerated in sanitized tanks until further processing. After all of the broth is column purified, the batches of protein produced are pooled and condensed to reduce volume. Before drying the condense protein is microfiltered to assure eliminate the potential for microbiological contamination. The pH is reduced to 6.0-6.2 with diluted acetic acid and sent for freeze drying by a contracted pharmaceutical grade manufacturer.

1.6. Uses and Natural Occurrence

In nature, Apoaequorin occurs as a single polypeptide chain protein, produced by specialized tissues of the jellyfish, *Aequorea victoria*. In the jellyfish, the protein Apoaequorin is produced and then conjugated to a small prosthetic group, coelenterazine, to create the bio-luminescent protein Aequorin. The protein is an enzyme when charged with its prosthetic group; specifically it is an oxygenase and, upon binding calcium it hydrolyzes a carbonyl group of coelenterazine in the presence of oxygen. The reaction releases carbon dioxide and light (Inouye et al., 1989; Inouye and Sakaki, 2006). In the animal, the light is captured by the green fluorescent protein or GFP, which releases the energy of the blue photon at a lower energy level by fluorescence. This causes a burst of green light to be released from the penumbral ring of the bell of the jellyfish. This photoluminescence by Apoaequorin only occurs when the protein is combined with its prosthetic group, a luciferin-type molecule, termed coelenterazine which is obtained from the diet. In this form, Apoaequorin and coelenterazine combine to form the molecule called Aequorin. The protein has been used for over 40 years as a luminescent indicator to elucidate the role of calcium as a second messenger in cell signaling cascades (Kendall and Badminton, 1998). The protein has been found to be innocuous to all cells that have been subjected to many such investigations.

There also exists a history of human consumption of edible jellyfish and in turn likely exposure to the protein Apoaequorin. Jellyfish are a common food in the diet of Southeast Asian countries (Kitamura and Omori, 2010; Li and Hsieh, 2004). The harvesting of these coelenterates

is without regards to species identification but it is common knowledge that several species are captured for the food industry (Kitamura and Omori, 2010). Only DNA testing of the different types of jellyfish can distinguish the species of the catch. This is not performed by either the fishermen or the industry in general. It is uncertain what other jellyfish could be included in the harvest as most harvests are based upon seasonal blooms. There is however a general interest in increasing the jellyfish harvests to include several species both for their health benefits as well as a growing need to create larger protein resources for the global population growth and because jellyfish blooms are becoming much larger for as of yet undetermined reasons (Richardson et al., 2009; Peggy Hsieh et al., 2001). Harvesting of jellyfish is considered to be a practical and profitable means by which to manage the ecology of these regions of the world (Peggy Hsieh et al., 2001). In recent years demands for edible jellyfish has increased in Southeast Asia (Kitamura and Omori, 2010). Although it is difficult to identify the dietary exposure to luminescent jellyfish or similar species, based on the considerations above, it is likely that humans are exposed to Apoaequorin.

The available information shows that apoaequorin is endogenous to a number of pelagic invertebrates, the most notable of which is the jellyfish *Aequorea victoria* for which the protein is named. The protein is produced and localized in specialized organs of the circumferential ring of the bell of the jellyfish. All jellyfish are invertebrate animals belonging to the phylum Cnidaria (formerly Coelenterata) and are unrelated to fish, which are vertebrates. The Cnidarians are a group of aquatic animals that include the jellyfish, corals, sea anemones and hydras. They are radially symmetrical in body plan with a single opening for a mouth and waste release that is surrounded by tentacles. They possess two stages in life the medusa and the polyp. The medusa stage is typified by the shape of a jellyfish. These animals have no brain or bones and move by undulations of the gelatinous bell of the medusa stage.

It should be noted that bioluminescence is not expected to occur when apoaequorin preparation is added to the proposed foods (milkshakes and smoothies). The ability of apoaequorin to luminesce is dependent on conjugation with a cofactor called coelenterazine. The holo-enzyme or holozyme is then termed aequorin. This molecule is found in the jellyfish in the wild and formed in the lab for those applications where luminescence is the aim of utility. However Quincy's Apoaequorin cannot and will not luminesce since the cofactor is not present.

1.7. Intended Use Levels and Food Categories

Apoaequorin is intended for use as a food ingredient at levels up to 6 mg of Apoaequorin *per* serving (reference amounts customarily consumed, 21 CFR § 101.12) in dairy products such as ice cream/ice milk (milk shake) and fruit juices (smoothies) (Table 6) when not precluded by a standard of identity.

1.7.1. Estimated Daily Intake from the Intended Uses

The possible daily intake of Apoaequorin protein preparation is estimated using "maximum" intended use levels of Apoaequorin in ice cream/ice milk (including milkshake) and fruit juices and consumption of these food products/categories. Based on USDA CSFII surveys (Smiciklas-Wright et al., 2002) for quantities of foods consumed daily, the mean and 90th percentile consumption of Apoaequorin from the proposed uses in ice cream/ice milk

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(milkshake) and fruit juices (smoothies) was estimated (Table 6). This database provides estimates of quantities consumed by users of specified foods during an eating occasion and during a day. The CSFII data provides intake of ice cream/ice milk that is likely to include milkshake and it also provides intake of fruit juices that is likely to include smoothies. Hence, in Table 6, values for ice cream/ice milk and fruit juices are included. The intended use of Apoaequorin at levels of 6 mg of *per* serving will result in mean and 90th percentile intake of 8.94 and 19.05 mg/person/day, respectively. For an individual weighing 60 kg the resulting intake will be 0.33 mg/kg bw/day. For safety assessment purposes, the maximum intake of 20 mg/person/day (0.33 mg/kg bw/day) of Apoaequorin is considered as appropriate.

Table 6. Intended Use Levels and Possible Daily Intake of Apoaequorin Based on USDA Data[1]

Food category	Consumption of food product (g/day)		Use levels/ serving (mg)	Use levels (mg/kg)	Average serving size (g)	Daily intake by adult (mg/person)	
	Mean	90th %				Mean	90th %
Protein shakes[2]	151	266	6	25	240	3.77	6.65
Smoothies[3]	207	496	6	25	240	5.17	12.40
					Total (µg/person/day)	**8.94**	**19.05**

[1]The daily intake calculations are based on USDA data and mean portion size (21 CFR §101.12). [2]For protein shakes, ice cream/ice milk is considered; [3]For smoothies, fruit juices- apple juice intake is considered to represent smoothies.

2. TOXICOLOGY

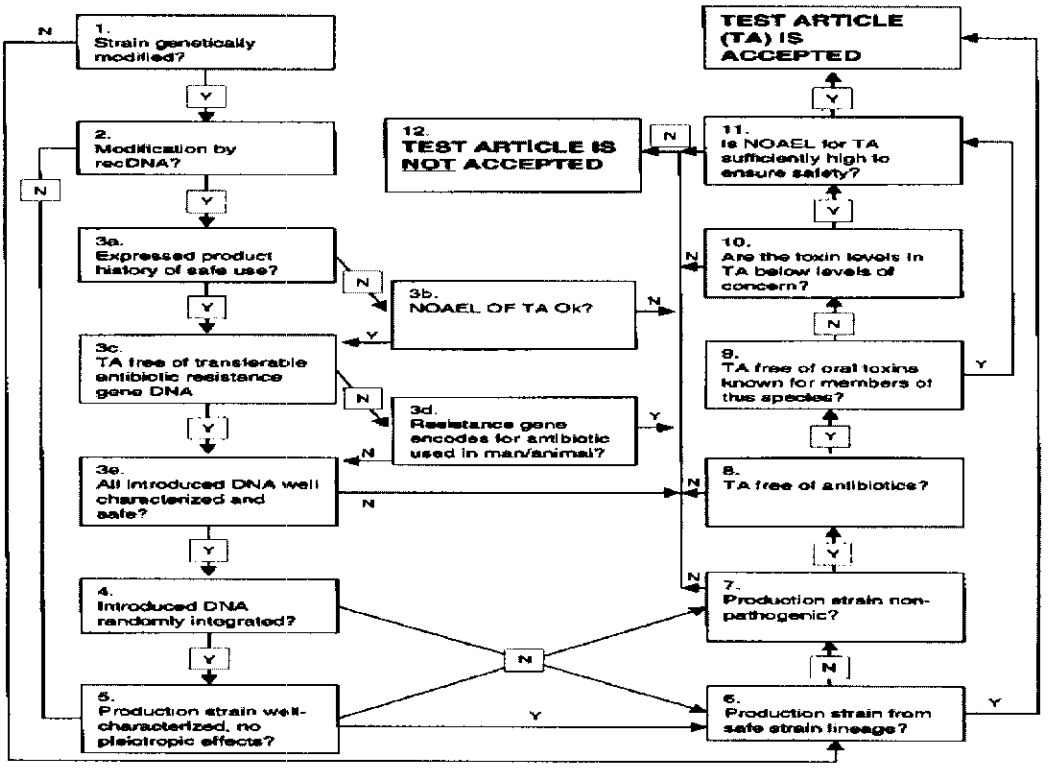
2.1. Evaluation by the Pariza and Johnson Decision Tree

Although, the decision tree outlined by Pariza and Johnson (2001) is commonly used to evaluate the safety of microbial enzyme preparations intended for use as processing aids, it can be used for the safety of other similar food ingredient. The decision tree begins by asking whether or not the production strain is genetically modified. As the production strain of this GRAS ingredient has been genetically modified using rDNA techniques, one must answer a number of questions relating to the introduced DNA. Apoaequorin meets the minimal criteria for assessment of an engineered protein to be safe for human consumption described in the decision tree diagram (Figure 2) by Pariza and Johnson (2001). The fact that the assessment Step 3a (Figure 2) cannot be ascertained without a direct history of human consumption at the concentrations resulting from the intended uses of Apoaequorin described in this GRAS assessment, a NOAEL assessment would be required for assessing the safety of the protein produced by recombinant means. As discussed below the 13-week oral toxicity study provides a sufficiently high (>2000-fold) safety factor to ensure safety. The test article is free of transferable antibiotic resistance DNA; no antibiotic resistance markers were used during the genetic modification steps used to obtain the *E. coli* production microorganism. All other introduced DNA is well characterized and free of attributes that would render it unsafe for constructing microorganisms to be used to produce food-grade products.

Question 4 of the decision tree asks if the introduced DNA is randomly integrated into the chromosome, and if so, whether the production strain is sufficiently characterized so that one

can make reasonable assumptions about unintended pleiotropic effects. There is a single copy of the gene inserted into the chromosome. There are no fragments, no vector, and no antibiotic resistance gene. The toxicity studies performed using the Apoaequorin test preparations derived from the *E. coli* production microorganism did not uncover any treatment related toxicity, or induction of gene mutation. Thus it is reasonable to assume that no unintended pleiotropic effects which could result in the synthesis of toxins or other unsafe metabolites have occurred due to the genetic modification that was employed. The production strain is from a known safe strain lineage. Thus it can be assumed that the production microorganism is unlikely to cause pathogenicity and antibiotic production. The results of the 13-week dietary study showed that the NOAEL for Apoaequorin preparation was sufficiently high to ensure safety. With these questions answered, the decision tree has been navigated to the point where the product can be accepted.

Figure 2. Decision tree analysis for safe protein assessment (Pariza and Johnson, 2001)



2.2. Mutagenicity Test

2.2.1. Ames Assay

The potential of lyophilized Apoaequorin protein (powder) preparation to induce gene mutations (Ames assay) in comparison to vehicle control was investigated in a bacterial reverse

mutation assay using *Salmonella typhimurium* strains TA98, TA 100, TA 1535, TA 1537 and tester strain *Escherichia coli* WP2 uvrA (Bioservice, 2009). The study was conducted at BSL Bioservice Lab in Germany and in compliance with OECD guidelines for Testing of Chemicals (9th Edition), the Commission Regulation (No 440/2008), the EPA Health Effects Test Guidelines and BSL Bioservice standard operating procedures. The assay was performed in two independent experiments (plate incorporation and pre-incubation test) both with and without rat liver microsomal activation. The test compound concentration used in these experiments were 31.6, 100, 316, 1000, 2500 and 5000 µg/plate. In addition to test compound, negative (vehicle DMSO) and positive control (sodium azide, 4-nitro-o-phenylene-diamine, methylmethane sulfonate, and 2-aminoanthracene) groups as per assay requirements were included.

In both experiments, in the presence or absence of metabolic activation, no precipitation or toxic effects of Apoaequorin protein preparation was noted in any of the tester strain. The reduction in the number of revertants down to a mutation factor of 0.5 found in pre-incubation experiment in *S. typhimurium* tester strain TA100 at a concentration of 316 g/plate (in the absence of metabolic activation) and in tester strain TA 1537 at a concentration of 316 µg/plate (in the presence of metabolic activation) was regarded as not biologically relevant due to lack of a dose response relationship. The results of these experiments (plate incorporation and pre-incubation test) did not reveal biologically relevant increases in revertant colony numbers of the any of the five tester strains of *S. typhimurium* or *E. coli* WP2 uvrA following treatment with lyophilized Apoaequorin protein preparation at any of the concentrations levels tested in the presence or absence of metabolic activation. The results of reference mutagens (positive control) revealed a distinct increase in reveratant colonies supporting the validity of the experiments. The observation from these experiments suggest that under the experimental condition lyophilized Apoaequorin protein preparation is unlikely to cause gene mutations by base-pair changes or frameshifts in the genome of the tester strains used. Thus, Apoaequorin protein is considered as non-mutagenic in bacterial reverse mutation assay (Bioservice, 2009).

2.3. Animal studies

2.3.1. Subchronic Toxicity Study

In a dose-response study in rats, Moran et al. (2013) investigated the potential toxicity of Apoaequorin protein preparation (Prevagen®) in rats. The study was conducted in accordance with OECD and FDA Redbook (2000) guidelines for such studies. Apoaequorin protein preparation was administered via oral gavage at dose levels of 0 (control; Group I), 92.6 (Group II- low dose), 462.9 (Group III- mid dose), or 926.0 (Group IV- high dose) mg/kg bw/day to Sprague-Dawley rats (10/sex/group) for at least 90 consecutive days. Two additional groups of animals for the recovery study received 0 (Group V) and 926 (Group VI) mg/kg bw/day of the protein preparation for 90 days, followed by no additional treatment for 28 days. The active ingredient (Apoaequorin protein) content of the test substance was 72%. Thus the equivalent dose of Apoaequorin protein received by each group was 0, 66.7, 333.3, and 666.7 mg/kg bw/day, respectively. These dose levels targeted approximately 200, 1000, and 2000 times the expected human oral intake on a 20 mg/day basis. The highest dose administered to animals was determined as 2000 fold higher than a human dosage at 20 mg protein/person (60 kg bw).

Throughout the study period the animals were observed for clinical signs of toxicity (once daily) and mortality (twice daily), while detailed clinical examinations, and body weight

and feed consumption were recorded weekly. Ophthalmological examination was performed on all animals before the start of study and on Day 90 of the study. At termination, urinalysis, hematological, and clinical chemistry parameters were investigated. Organ weights were recorded at termination and over 40 tissues and organs were harvested at the necropsy and fixed in 10% neutral buffered neutral formalin. Histopathological examination was carried out on the full set of tissues collected from the high dose and control groups. All gross lesions from all rats irrespective of group were also evaluated for histological changes (Moran et al., 2013).

There were no test article-related or other mortalities noted during the course of the study. There were no clinical signs in either male or female animals resulting from the administration of Apoaequorin protein preparation. All clinical signs and observations were considered incidental and largely resolved during the course of study or by the end of the treatment phase. Ophthalmoscopy prior to study initiation and near experimental completion (Day 90) did not reveal any treatment-related abnormalities. The mean weekly body weights and feed consumption of treatment groups were comparable to the control groups throughout the treatment period. Some sporadic minor but statistically significant changes in body weights as well as feed consumption noted at a few weekly measurements were considered incidental and unassociated with test article administration (Moran et al., 2013).

There were no treatment-related adverse effects noted in urinalysis parameters in male and female rats in the 90 day study groups or in the recovery group. Similarly, there were no treatment-related biologically significant effects of the Apoaequorin protein treatment in hematology parameters in male and female rats. Few hematology changes such as statistically significant increase red cell distribution width in male rats (Group IV), absolute immature lymphocytes (one female each in Groups II, and III, two females revealed absolute atypical lymphocytes (Group III) and one of these animals also exhibited absolute hypersegmented nutrophils, decrease in prothrombin time(Group II and III) were noted. These changes in hematology parameters were not observed in both sexes, lacked correlating changes in other clinical parameters, were of small magnitude, were not noted in a dose-related manner, or were not associated with microscopic changes in the related organs. Accordingly, they were considered as incidental changes/biological variations and not treatment-related adverse effects. There were no treatment-related statistically significant effects of the Apoaequorin protein preparation in serum chemistry parameters in male and female rats. The serological analysis did not reveal any detectable titers against the usual battery of prospective pathogens and antigens tested (Moran et al., 2013).

As compared to the vehicle control group, treatment with Apoaequorin protein preparation did not results in toxicologically significant changes in absolute and relative organ weights. However, in Group III females, absolute heart weight and heart-to-brain weight ratio were statistically increased. This increase in heart weight was considered as incidental and toxicologically insignificant due to lack of dose-dependency and lack of correlating changes in clinical chemistry and histopathology. At scheduled necropsy there were no treatment-related gross pathological observations in any of the groups of rats following administration of the test article, Apoaequorin protein. Among the incidental changes noted included, the uterus/oviducts were noted as fluid-filled in 5 of 10 Group I animals at 0 mg/kg/day, in 4 of 10 Group II animals at 92.6 mg/kg/day, in 1 of 10 Group III animals at 462.9 mg/kg/day, and in 3 of 10 Group IV animals at 926.0 mg/kg/day of Apoaequorin. Microscopically, fluid-filled uteri usually corresponded microscopically to luminal dilation of the uterus that was attributable to variation

in the estrus cycle in individual animals, a finding not associated with test article administration. Additional macroscopic observations were of sporadic incidence and showed no trends/patterns to suggest a relationship to administration of Apoaequorin protein (Moran et al., 2013).

There were no microscopic findings related to the administration of Apoaequorin protein preparation. In the kidneys, there was minimal chronic progressive nephropathy (CPN) usually of minimal intensity in 9 of 10 males and 2 of 10 females of control group, as well as in 8 of 10 males and 4 of 10 females of high dose group (Moran et al., 2013). CPN was characterized by limited foci of tubular basophilia, thickened tubular basement membranes, tubular casts, and/or mononuclear cell infiltrates that affected less than 1% of nephrons. CPN at this low level intensity with the observed incidence pattern was a background finding unrelated to the test article administration. As observed in this study, CPN is an incidental finding that occurs predominantly in males, occurs in Sprague-Dawley rats with increased incidence as compared to other rat strains, and can be seen histologically as early as two months of age (Percy and Barthold, 2007; Hard and Khan, 2004). Additionally, there were a few microscopic observations of minimal to moderate intensity that were observed in single to a few males and/or females in Group IV. These observations included chronic inflammation of the Harderian gland, coronary arteritis and cardiomyopathy, neutrophil infiltrates of the pancreas, ulcer of the forestomach, gastric squamous cyst, acute inflammation of the thyroid gland capsule (unilateral), and hepatocellular vacuolation. These limited findings were considered incidental and toxicologically irrelevant as there were no convincing incidence patterns/trends to suggest a relationship to test substance administration. The remaining microscopic findings were not test article-related and were incidental as can be observed in the age and strain of rats used in this study (Percy and Barthold, 2007).

Based on the results of this subchronic toxicity study, the NOAEL for Apoaequorin protein preparation was determined as 926 mg/kg bw/day, the highest dose tested (Moran et al., 2013). As the protein preparation used in the study contained approximately 72% Apoaequorin, the resulting NOAEL for Apoaequorin will be 666.7 mg/kg bw/day, the highest dose tested.

2.4. Allergenicity and Safety

As mentioned earlier, bioluminescence has revolutionized the research into many cellular and molecular-biological processes, ranging from intracellular signaling to gene transcription (Kendall and Badminto, 1998). For over 4 decades, photoprotein Apoaequorin has been extensively used as a bioluminescence analytical tool in laboratories around the world to understand biochemical mechanisms, primarily in bacterial and eukaryotic cultures, transgenic systems including plant and animal transformations and even embryo studies. Injections of the protein as wells as transient and stable transformation of expression cassettes into living cells have been performed on the simplest organisms such as cyanobacteria to fish embryos and transgenic mice without any suggestion of adverse reaction due to the presence of Apoaequorin in the cells of these living systems (Torrecilla et al., 2002; Cheung et al., 2006; Rogers et al., 2007).

In order to assess any potential allergic reaction arising from Apoaequorin, a series of assessments were undertaken. These assessments for the protein in regards to allergenicity primarily relied on three significant studies following the Codex Alimnetarius Guidelines (2003). The first study entailed the examination of peer reviewed literature published by experts in the

field of toxicology and immunology, particularly as it pertains to allergenicity testing of proteins and any literary evidence of known allergenicity with the animal from which the gene was derived, i.e., *Aequorea victoria*. The second study presented is an *in silico* assessment of this Apoaequorin amino acid sequence alignment to known allergens through an extensive bioinformatics search of several key databases developed for this work. The third study is based on *in vitro* digestion studies which simulate the conditions of ingestion of the protein and its likely disposition in the gastric environment of the stomach. These studies on the assessment of Apoaequorin's allergenic potential were conducted at the Food Allergy Research and Resource Program in the Food Science and Technology Department of the University of Nebraska. The full allergenicity and *in vitro* study reports are enclosed as Appendix IV and V, respectively.

2.4.1. Fermentation Medium and Allergenicity

The production of the apoaequorin protein is by a recombinant fermentation and requires the use of amino acids as precursors to protein synthesis. The source of these nitrogen precursors is from the digested proteins of yeast extract, soybean meal and milk protein or casein. The seed tank (800 L) used to inoculate the working fermentation medium uses 10 g/L of yeast extract and 10 g/L of soy digest. The production fermentation uses up to 10 g/L hydrolyzed casein and 10 g/L yeast extract. Soy and casein are known allergens for a small percent of the human population.

Commercial suppliers modify these raw material sources through an acid hydrolysis of the protein constituents, effectively destroying the potential allergenicity of the protein constituents comprising the bulk of the raw material; converting the proteins into amino acids and peptides. In each stage of growing the production strain, the constituent amino acids are used to build biomass as in the early stages of fermentation or they are used to produce the apoaequorin protein; the strain acting as a catalyst for this conversion of monomers into polypeptide chains to produce the protein. The production strain is known not to be allergenic to humans. The apoaequorin protein has been shown through our adherence to the testing guidelines of the Codex (2003) to have no allergenic potential.

Any questions on the possible carryover of unspent peptide fragments from the hydrolyzed casein or soy materials that could, in theory, remain allergenic are answered by downstream processing of the clarified protein. In downstream processing the protein is removed from the spent medium components by capture on anion (negative charge) exchange chromatography. This technique effectively captures highly negatively charged proteins from a liquid carrier and retains the protein, as the medium passes through the column in the waste stream. The captured protein is then washed free of any residual components not bound by the chromatography resin. The protein is then eluted from off the column using a high salt solution for further processing.

Several means are used to "visualize" the purification stream in order to confirm recovery, concentration and residual contaminants. Of these techniques fractionation of a purified sample on SDS PAGE followed by protein staining has shown that the resulting protein obtained from purification is free of other protein/peptide molecules. After the protein is dried to a powder it is blended into a powdered and flavored milk shake with ingredients composed of milk and soy products. While these production and processing steps assure that the protein for commercial sales is free of any potential allergic ingredients it is blended into food grade ingredients that are composed of milk and soy products. The final commercial packaging is clearly labeled with this

information to alert the consumer of potential allergenic risks in preparing and consuming the product.

Lactose is a milk sugar used in the production of the apoaequorin protein as well. Lactose is not digested by the majority of adults in the human population causing lactose intolerance. This milk sugar is a sterilized addition to the post cook fermentation and is hydrolyzed to glucose and galactose sugars by the production strain. It is rapidly consumed by the organism and disappears from the fermentation broth within 15 minutes of the strain's demand for the sugar. No residual lactose is found in the fermentation broth. The purpose of the addition of lactose is to induce the strain to produce the protein, apoaequorin. This induction slows biological growth and triggers gene expression of the recombinant expression cassette.

2.4.2. Source Allergenicity

In order to assess the safety of Apoaequorin in the human diet, Codex Alimetrius recommendations were considered. From this source it is clear that animal toxicological studies would only be pertinent to risk assessment of proteins as a category if the test article were derived from a gene sequence obtained from a species known to induce toxicity or allergenicity. Therefore, considering the source of the gene sequence from which a codon optimized form of the gene was developed for expression in host cell types, *Aequorea victoria* is neither a known allergen nor a toxic coelenterate to human beings. The database searches using the organism name (*A. victoria*) or the family name (Aequoreidae) did not reveal any article-related to allergic effects of the organism. Thus, the available information from published literature indicates that Apoaequorin protein is unlikely to be allergenic.

2.4.3. Bioinformatics Analysis

The online database maintained at the Food Allergy Research and Resource Program, University of Nebraska was screened for alignment scores using he FASTA3 algorithm using the default scoring matrix (BLOSUM 50), looking for matches in alignment of low E score values (<1e-7) and greater than 50% identity. A sliding window of 80 amino acids for the sequence was further performed to find identities in the same way. Matches identified by such searches would be expected to be assayed by human serum testing using sera from specific allergic donors to see cross reactivity. The results obtained from both searches were negative making sera cross reactivity studies unnecessary. Additionally, a search of the Apoaequorin sequence for any eight contiguous amino acid identity match to any known allergen in the database was performed. This search was also negative for matches (Appendix V).

Furthermore, the Apoaequorin protein sequence was also searched against the non-redundant NCBI data set using BLASTP to screen against protein function and extend the screen for potential safety issues. Apoaequorin, a Ca2+ binding protein, is closely related to similar protein from other hydrozoans of phylum Cnidaria with E scores smaller than 10^{-40} and identifies >60% over the full length. None of these proteins have been identified as allergic. An additional BLASTP search using the keyword limit of "allerg*" failed to identify any match to allergenic sequence in NCBI with an *E* score smaller than 10^{-3} or a sequence homology of > 35% identity. These observations did not reveal any evidence to suggest that the Apoaequorin protein is an allergen or would present a possible risk of cross-reactivity to those with known allergies (Appendix IV).

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2.4.4. *In vitro* Protein Digestion

This allergenicity assessment includes testing the stability of the Apoaequorin protein in a pepsin digestion assay to help evaluate the probability that the protein may sensitize individuals leading to a food allergy. Ofori-Anti et al. (2008) suggested that slow digestibility of food proteins by the gastric protease, pepsin, is a characteristic commonly found with food allergens and recommended use of this assay as a simple tool for evaluating the potential risk that a new dietary protein might become an important food allergen when there is not a substantial, clear history of consumption and of allergenicity of the protein. As described earlier (see Section 2.2), Apoaequorin protein was subjected to digestions using the method described by Ofori-Anti et al. (2008).

The results of this study revealed that Apoaequorin protein was rapidly digested in pepsin at both pH 1.2 and 2.0. The SDS-PAGE colloidal blue gel staining method used to measure residual Apoaequorin protein revealed that more than 90% of the protein was digested in less than 30 seconds. At this time point, residual degradation bands were noted except for a very faint smeared band at approximately 3.5kDa that also nearly disappeared by 120 seconds. The assays demonstrated good reproducibility. The results of this experiment suggest that Apoaequorin is rapidly degraded in pepsin at acidic pH. These observations along with Codex (2003) guidelines indicate that there would not be a concern of potential allergenicity following human exposure based on stability of the protein in pepsin. The results of this study also suggest that the digestion characteristics of the Apoaequorin are similar to those of common non-allergenic dietary proteins (Appendix IV).

2.5. Other Safety Related Information

2.5.1. Human Clinical Studies

In a double-blind, placebo-controlled clinical trial, Underwood et al. (2011) investigated the effects of an Apoaequorin dietary supplement on specific areas of cognitive functioning using quantitative, computerized assessments. Although the primary objective of this trial was to examine changes in cognitive function, the study also provided a direct opportunity to observe the safety and 'tolerability' of Apoaequorin. In this study, a total of 218 healthy participants, aged 40 to 91 years (148 females; 70 males; 2 subjects lost) were randomly assigned to daily receive either Apoaequorin (10 mg) or placebo for three months duration. The placebo arm consisted of 92 subjects, while the Apoaequorin arm included 126 subjects. Participants were tested at 8, 30 and 90 day time points using quantitative, computer-based cognitive tasks of maze learning and recall (CogState Ltd). The Apoaequorin arm showed a statistically significant decrease in the number of errors, and increase in accuracy of performance. Only two participants dropped out of the study because of an adverse reaction, one each from the Apoaequorin and placebo arms. In this 3 month study, daily intake of Apoaequorin at a dosage of 10 mg per day was well tolerated by the study participants with no reported adverse reactions (Underwood et al., 2011).

In a single published series, four patients (3 male, 1 female, ages 35-52 years) with sporadic amyotrophic lateral sclerosis (ALS) received Apoaequorin along with CoQ10, Noni juice, turmeric extract, deprenyl, intravenous glutathione, lithium and a diet rich in medium chain triglycerides (Payne, 2009). In this study, the patients received one twenty milligram (20

mg) capsule every 2-3 hours during waking hours and one to two capsules 30-60 minute before retiring for the night for about 2 years (not mentioned how long the patients received Apoaequorin). As reported in the publication, all four patients "experienced disease progression. However when compared to age and disease matched controls by the author the degree of progression is decidedly less." Additional details of the study, including any adverse effects were not reported.

2.5.2. Dietary Supplement Use & Adverse Events Reports

Apoaequorin protein preparation manufactured as Prevagen® has been marketed as a dietary supplement, since 2007. Quincy has put in place a procedure to monitor and systematically review all the adverse events received. As of January 2014, Quincy received approximately 2,200 adverse event reports (Quincy Bioscience, 2014). These reported events included headache, fainting, dizziness, nausea, chest pain, seizures, tremors, and allergic reactions like itching, skin rashes. Quincy categorized these reports as "non-serious" and "serious." Based upon the details of the individual complaint and as per the information in The National Cancer Institute-Common Terminology Criteria for Adverse Events -Version 4 (CTCAE 4.0- NCI, NIH Guidance document)[2], a severity grade for each Adverse Event is assigned. Of the 2,281 reports received, the top ten with their percentage were as follows: Headaches- 428 (18.76%); Dizziness- 172 (7.54%); Nausea 155- (6.80%); Hypertension- 84 (3.68%); Diarrhea- 74 (3.24%); Memory Impairment- 74; (3.24%); Insomnia- 63 (2.76%); Anxiety- 54 (2.37%); and Stomach Pain- 51 (2.24%). A majority of these reports were received via telephone call. Adverse Events with grade III or greater (CTCAE 4.0 criteria) were further investigated. A total of 26 reports were identified as serious adverse events. The serious events reported were almost all anecdotal and mainly neurologic or cardiovascular. Most of the events were associated with pre-existing chronic conditions and are attributable to those conditions. As required, serious events were reported to FDA. As purchases for Prevagen® were made through retail outlets, directly through Quincy's website, as well as through various other online retailers, it was difficult for Quincy to provide the number of consumers who bought the product. However, based on the Adverse Events Report summary sheet, Quincy estimated that 1,963,467 bottles of Prevagen® were sold until January 2014.

In order to gain further insight on the association between the adverse event and any role of Apoaequorin protein preparation (Prevagen®) in the causation of the adverse event, two independent expert physicians (Dr. Williams and Dr. Pi-Sunyer) were retained by Amin Talati, LLC (a law firm representing Quincy) to review the serious adverse event reports, as well as a number of non-serious adverse event reports. Both physicians concluded that Apoaequorin protein preparation (Prevagen®) was unlikely to have caused the serious adverse events reported. Reports from these physicians are provided as Appendix VI and VII. Additionally, Dr. Williams and Dr. Pi-Sunyer also served as advisors to the Expert Panelists. A letter from Amin Talati, LLC with additional information regarding Quincy's investigation of adverse events reported by consumers of the Prevagen® supplement product containing apoaequorin as well as summarizing the physician's findings, is attached as Appendix VIII.

[2] Common Terminology Criteria for Adverse Events (CTCAE) Version 4.0. Available at: http://evs.nci.nih.gov/ftp1/CTCAE/CTCAE_4.03_2010-06-14_QuickReference_5x7.pdf

2.5.3. Metabolism

In an unpublished *in vitro* study, Apoaequorin protein was subjected to digestion (metabolism) in simulated gastric fluid (SFG) using pepsin enzyme from porcine (Goodman and Ofori-Anti, 2010). The SGF preparation and digestion procedures were based on the methods described by Ofori-Anti et al. (2008). In this study, SDS-PAGE colloidal blue gel staining method was used to measure residual protein. At two different pH (1.2 and 2.0) SGF solutions used in this assay, Apoaequorin protein was found to be rapidly digested by pepsin. The SDS-PAGE measurement revealed that within 30 seconds, over 90% of the Apoaequorin protein was digested or hydrolyzed. At the 30 second time point, no residual degradation bands were detected except for a very faint smeared band at approximately 3.5 kDa that also almost completely disappeared by 120 seconds. The results of this experiment suggest that Apoaequorin is rapidly degraded by pepsin at acidic pH. These observations suggest that, similar to other common dietary proteins, Apoaequorin is digested or enzymatically hydrolyzed to individual amino acids that can be absorbed in the digestive tract (Appendix IV).

2.5.4. Inherent Characteristics

Apoaequorin is a small calcium-binding protein with properties typical of globular proteins. It possesses features typical of EF-hand[3] binding proteins; being composed of amino acids important to human metabolism. Apoaequorin has been used for over 40 years in living systems as an analytical tool for cell biology without evidence of adverse biological effects. Apoaequorin shares homologies with calcium-binding proteins also found in the human body (Matsuo and Kanehisa, 1993; Zhou et al., 2006) suggesting that it is unlikely to cause adverse effects. Apoaequorin, produced using recombinant technology, consists of 196 amino acid residues and is identical to naturally found Apoaequorin protein in jellyfish. As discussed earlier, historic account of human exposure to the edible jellyfish, and in turn, exposure to the protein Apoaequorin, provides anecdotal evidence of its safety. Additionally, as a polypeptide with 196 amino acids that are common components of dietary proteins, Apoaequorin is likely to be metabolized similar to other dietary proteins and is unlikely to cause any adverse effects.

2.5.5. Calcium Binding Properties

Given the calcium binding properties of the aequorin photoprotein, it is important to address whether the intended uses of apoaequorin preparation will impact calcium bioavailability in the target foods. To address this, the amount of calcium that would be displaced (maximally) from a serving of milk (or water) in a prepared smoothie or shake was calculated. The calcium binding capacity of apoaequorin was determined by a molar ratio of 3 calcium ions to 1 calcium-binding protein. The molecular weight of calcium is 40.1 g/mole and the molecular weight of apoaequorin is 22,300 g/mole. Based on this amount of calcium bound to the protein at different levels of apoaequorin (5, 10 and 20 mg) was calculated (Table 7). The findings from these calculations suggest that the amount of calcium bound will be very small (negligible) and is unlikely to impact calcium bioavailability in target foods. Also compared to this value the daily recommended allowance of 1200 mg calcium is over 10,000 fold higher.

[3] The EF hand is a helix-loop-helix structural domain found in a large family of calcium-binding proteins.

Table 7. Amount of Calcium Utilized by Apoaequorin protein

Amount of apoaequorin	Moles of apoaequorin	Moles of calcium	Amount of calcium
5 mg	0.224 µmoles	0.673 µmoles	**27 µg**
10 mg	0.448 µmoles	1.346 µmoles	**54 µg**
20 mg	0.896 µmoles	2.692 µmoles	**108 µg**

3. SUMMARY AND DISCUSSION

Quincy Bioscience intends to use Apoaequorin, a standardized protein preparation (Prevagen®) produced by recombinant DNA technology for use as a food ingredient in selected foods. Apoaequorin protein preparation is a light tan colored powder without any appreciable odor. The production of Apoaequorin is achieved by fusing the Apoaequorin cDNA to the signal peptide coding sequence, *pelB,* which targets the periplasmic space of the cell. The production organism, *E. coli* BL21 (DE3) is nonpathogenic and nontoxigenic, and the protein preparation is made according to current GMP. The DNA introduced into the *E. coli* BL21 (DE3) recipient strain is well characterized and shown to be safe. The production process is fully characterized and standardized. The protein powder is standardized to contain ~50-75% apoaequorin protein, 25-50% sodium chloride, and minor components of the purification buffer such as acetic acid (<7%). The protein molecule of the preparation is a single polypeptide chain constituted of 196 amino acids and is rich in aromatic amino acids such as tryptophan, (3%), tyrosine (4%) and phenylalanine (4%). Quincy Bioscience intends to use Apoaequorin at levels up to 6 mg/serving (reference amounts customarily consumed, 21 CFR § 101.12) in ice cream/ice milk (milkshake) and fruit juices (smoothies). The intended use of Apoaequorin protein preparation in the above mentioned food categories will result in a mean and 90th percentile estimated daily intake of 8.94 and 19.05 mg Apoaequorin/person/day, respectively.

Apoaequorin produced by Quincy Bioscience is identical to the naturally occurring protein present in jellyfish *A. victoria.* In nature, Apoaequorin is produced by specialized tissues of jellyfish. The protein has been used for over 40 years as a luminescent indicator to elucidate the biological role of calcium as a second messenger in cell signaling cascades and is considered as innocuous to all cells that have been subjected to such investigations. In eastern cultures, some species of jellyfish are commonly consumed by humans as food. Although it is difficult to identify the dietary exposure to luminescent jellyfish or similar species, it is likely that humans are exposed to Apoaequorin.

The findings from an *in vitro* study suggest that Apoaequorin dissolved in simulated gastric fluid is rapidly digested by pepsin enzyme. In less than 30 seconds, over 90% of Apoaequorin was digested and by 2 minutes almost all of the protein is digested. This suggests that following oral consumption by humans, Apoaequorin is likely to be completely hydrolyzed to individual amino acids that will be absorbed in a process similar to other dietary proteins. In a double-blind, placebo-controlled clinical trial, daily oral ingestion of Apoaequorin by adult human subjects (n=126) at a dose level of 10 mg/day for 90 days was well tolerated by the study participants with no reported adverse reactions. In a dose-response subchronic toxicity study (conducted as per OECD and FDA guidelines), gavage administration of Apoaequorin preparation to rats at dose levels up to 926.0 mg/kg bw/day (666.7 mg Apoaequorin/kg bw/day) for 90 days did not result in any mortality. In this study, there were no clinical or

ophthalmological signs, body weight, body weight gain, food consumption, food efficiency, clinical pathology or histopathological changes attributable to administration of Apoaequorin. Based on the results of this study, the NOAEL for Apoaequorin was determined as 667 mg/kg bw/day, the highest dose tested. In an Ames mutagenicity assay, Apoaequorin protein preparation was non-mutagenic.

Use of Apoaequorin protein as a biochemical luminescence analytical tool in different organisms such as bacteria, fish embryos and transgenic mice did not reveal any adverse reaction due to the presence of Apoaequorin in the cells of these living systems. In a series of assessments conducted as per Codex Alimnetarius Guidelines, allergnicity potentials of Apoaequorin protein based on extensive search of literature in the field of toxicology and immunology, *in silico* Apoaequorin protein amino acid sequence alignment to known allergens (bioinformatic search), and *in vitro* digestion studies in the gastric environment of the stomach were studied. The findings from these investigations suggest that Apoaequorin is unlikely to be allergenic. The safety evaluation of the proposed uses of Apoaequorin preparations by commonly used decision tree as outlined by Pariza and Johnson (2001) for microbial enzymes supports the safety-in-use. Published and unpublished information as described supports the safety of the following: recipient strain, introduced genetic material, production microorganism, Apoaequorin and its use in food, manufacturing process, the final Apoaequorin preparation, and potential residues in food.

There is sufficient qualitative and quantitative scientific information to determine the safety-in-use of Apoaequorin protein for its proposed uses in the above mentioned applications. The safety data on Apoaequorin protein preparation includes subchronic toxicity studies in rats, *in vitro* genotoxicity study, lack of alleginicity potentials, *in vitro* enzymatic hydrolysis (metabolism), and human tolerance following oral ingestion in a clinical trial. Based on the results of the subchronic toxicity study, the NOAEL of Apoaequorin was determined as 666.7 mg/kg bw/day, the highest dose tested. Generally, Acceptable Daily Intakes (ADIs) are derived from a NOAEL determined from animal studies with considerations of uncertainty factors to account for variability and uncertainties. A factor of 10 may be introduced to account for the possible differences in responsiveness between humans and animals in prolonged exposure studies and a second factor of 10 may be used to account for variation in susceptibility among individuals in the human population. Thus a resultant uncertainty factor of 100 has been judged to be appropriate. Applying this factor to the above NOAEL of 666.7 mg/kg bw/day will result in Acceptable Daily Intake (ADI) of 6.67 mg/kg bw/day. The estimated daily intake (20 mg/person/day or 0.33 mg/kg bw/day) from the intended uses of Apoaequorin is 11-fold lower as compared to the ADI determined on the basis of animal toxicity study. In addition to the specific safety study, other studies further support the safety of Apoaequorin at the intended use levels. The NOAEL for Apoaequorin of 666.7 mg/kg bw/day provides over 2000-fold safety factor over the estimated daily intake of 20 mg/person/day (0.33 mg/kg bw/day) for humans.

Quincy is marketing apoaequorin protein preparation (Prevagen®) as a dietary supplement since 2007 and has received over 2,200 adverse event reports. Of these events, 26 were categorized as serious events (Quincy defined, based on NCI, NIH guidance- CTCAE 4.0 criteria). The events reported were almost all anecdotal and were primarily neurologic or cardiovascular. The events did not reveal any commonality in presentation or duration of exposure preceeding event. Majority of the events were associated with pre-exisiting chronic conditions and are attributable to those conditions. A systematic and critical analysis of these events, as well as opinions of two Expert Physicians, Dr. Williams and Dr. Pi-Sunyer (also

served as advisers to GRAS Panel members), suggest that these events are unlikely to be associated with the ingestion of apoaequorin protein preparation (Prevagen®).

In summary, on the basis of scientific procedures[4], consumption of Apoaequorin protein preparation (Prevagen®) as a food ingredient at use levels of up to 6 mg/serving (reference amounts customarily consumed, 21CFR 101.12) in ice cream/ice milk (milkshake) and fruit juices (smoothies) is considered safe. The proposed uses are compatible with current regulations, *i.e.*, Apoaequorin protein preparation is used in specified foods (described in this document), when not otherwise precluded by a Standard of Identity, and is produced according to current good manufacturing practices (cGMP).

[4] 21 CFR §170.3 Definitions. (h) Scientific procedures include those human, animal, analytical, and other scientific studies, whether published or unpublished, appropriate to establish the safety of a substance.

4. CONCLUSION

Based on a critical scientific evaluation of the publicly available data summarized above, the Expert Panel members whose signatures appear below, have individually and collectively concluded that the Apoaequorin protein preparation, meeting the specifications cited here-in, and when used at a maximum use levels of up to at use levels of up to 6 mg/serving (reference amounts customarily consumed, 21 CFR § 101.12) in ice cream/ice milk (milkshake) and fruit juices (smoothies) as described in this GRAS monograph and resulting in the 90th percentile estimated intake of 20 mg of Apoaequorin/person/day (0.33 mg/kg body weight/day) is safe.

It is also our opinion that other qualified and competent scientists reviewing the same publicly available toxicological and safety information would reach the same conclusion. Therefore, we have also concluded that Apoaequorin protein preparation, when used as described, is GRAS.

Signatures

(b) (6)

28 Aug. 2014

John A. Thomas, Ph.D., F.A.C.T., D.A.T.S — Date

(b) (6)

August 25, 2014

Robert L. Martin, Ph.D. — Date

(b) (6)

27 Aug 2014

Michael W. Pariza, Ph.D. — Date

Advisers to the Expert Panel

(b) (6)

August 29, 2014

Madhusudan G. Soni, Ph.D., FACN, FATS — Date

(b) (6)

22 Aug 14

Gary M. Williams, M.D., DABT, FRCPath — Date

(b) (6)

8/21/14

Xavier Pi-Sunyer, M.D., MPH — Date

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6. APPENDIX I

Analytical data from five manufacturing batches

Specifications of Apoaequorin Protein Preparation from Five Non-consecutive Lots*

Parameter	Standard specifications**	Lot # 1203080	Lot # 1209399	Lot # 1209409	Lot # 1210429	Lot # 1105149***
Description	Light tan color powder	Complies	Complies	Complies	Complies	Complies
Apoaequorin content	50-60%	526 mg/g	530 mg/g	513 mg/g	586 mg/g	722 mg/g***
Identification	HPLC	Complies	Complies	Complies	Complies	Complies
Moisture	< 7%	2.70%	2.12%	1.60%	2.10%	1.81%
pH	5.5 - 6.5	6.21	6.47	6.50	5.96	6.05
Residue on ignition	25-35%	26.3%	32.4%	32.2%	34.90%	13.2%
Heavy metals						
Arsenic	<1 ppm	<10 ppb	16.4 ppb	14.5 ppb	11.5 ppb	<10 ppb
Cadmium	<1 ppm	<10 ppb	<10 ppb	<10 ppb	<10 ppb	<10 ppb
Lead	<1 ppm	8.96 ppb	<5 ppb	<5 ppb	5.33 ppb	15.1 ppb
Mercury	<2 ppm	<10 ppb	<10 ppb	<10 ppb	<10 ppb	11.2 ppb
Microbiological assays						
Aerobic plate count	<100,000 CFU/g	<10 CFU/g	10 CFU/g	<10 CFU/g	<10 CFU/g	<10 CFU/g
Coliform counts	<1000 CFU/g	<10 CFU/g	<10 CFU/g	<10 CFU/g	<10 CFU/g	<10 CFU/g
Escherichia coli	<10 CFU/g	<10 CFU/g	<10 CFU/g	<10 CFU/g	<10 CFU/g	<10 CFU/g
Staphylococcus aureus	<10 CFU/g	<10 CFU/g	<10 CFU/g	<10 CFU/g	<10 CFU/g	<10 CFU/g
Mold counts	<5000 CFU/g	<10 CFU/g	<10 CFU/g	<10 CFU/g	<10 CFU/g	<10 CFU/g
Yeast count	<5000 CFU/g	<10 CFU/g	<10 CFU/g	<10 CFU/g	<10 CFU/g	<10 CFU/g
Salmonella	Negative	Negative	Negative	Negative	Negative	Negative

*Based on information provided by Quincy Bioscience (2012); **Standard specifications for marketed product. ***Apoaequorin content of this lot was relatively high as a result of purification process- for future productions the content will be adjusted to remain within the standard specifications.

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7. APPENDIX II

Stability testing batch data

Pages 1-5

000048

QUINCY

2010 Pinehurst Drive, Middleton, WI 53562-2555

Stability Report

Material: Encapsulated Apoaequorin
Quincy Bioscience Lot No.: 804182

Manufacturer: Edan Naturals
Manufacture Date: April2008

TEST NAME	TEST METHOD	RESULT	DATE OF ANALYSIS
Assay (Apoaequorin/cap)	BCA	10.45 mg Apoaequorin / capsule	6May2008
Assay (Apoaequorin/cap)	BCA	9.79 mg Apoaequorin / capsule	8Sep2011

Approved by:
(b) (6)
29Jun2014
Mark Roeder
Technical and Quality Manager
Quincy Bioscience

000049

QUINCY

2010 Pinehurst Drive, Middleton, WI 53562-2555

Stability Report

Material: Encapsulated Apoaequorin
Quincy Bioscience Lot No.: 804175

Manufacturer: Edan Naturals
Manufacture Date: April2008

TEST NAME	TEST METHOD	RESULT	DATE OF ANALYSIS
Assay (Apoaequorin/cap)	BCA	10.45 mg Apoaequorin / capsule	30Apr2008
Assay (Apoaequorin/cap)	BCA	10.32 mg Apoaequorin / capsule	8Sep2011

Approved by:
(b) (6)
20Jun2014

Mark Roeder
Technical and Quality Manager
Quincy Bioscience

000050

QUINCY

2010 Pinehurst Drive, Middleton, WI 53562-2555

Stability Report

Material: Encapsulated Apoaequorin
Quincy Bioscience Lot No.: 804164A

Manufacturer: Edan Naturals
Manufacture Date: April2008

TEST NAME	TEST METHOD	RESULT	DATE OF ANALYSIS
Assay (Apoaequorin/cap)	BCA	10.05 mg Apoaequorin / capsule	30Apr2008
Assay (Apoaequorin/cap)	BCA	10.01 mg Apoaequorin / capsule	8Sep2011

Approved by:
(b) (6) 20Jun2014

Mark Roeder
Technical and Quality Manager
Quincy Bioscience

000051

QUINCY

2010 Pinehurst Drive, Middleton, WI 53562-2555

Stability Report

Material: Encapsulated Apoaequorin
Quincy Bioscience Lot No.: 803139

Manufacturer: Edan Naturals
Manufacture Date: March2008

TEST NAME	TEST METHOD	RESULT	DATE OF ANALYSIS
Assay (Apoaequorin/cap)	BCA	11.25 mg Apoaequorin / capsule	1Apr2008
Assay (Apoaequorin/cap)	BCA	11.33 mg Apoaequorin / capsule	8Sep2011

Approved by:
(b) (6)
20Jun2014

Mark Roeder
Technical and Quality Manager
Quincy Bioscience

000052

QUINCY

2010 Pinehurst Drive, Middleton, WI 53562-2555

Stability Report

Material: Encapsulated Apoaequorin
Quincy Bioscience Lot No.: 804183

Manufacturer: Edan Naturals
Manufacture Date: April2008

TEST NAME	TEST METHOD	RESULT	DATE OF ANALYSIS
Assay (Apoaequorin/cap)	BCA	11.06 mg Apoaequorin / capsule	6May2008
Assay (Apoaequorin/cap)	BCA	9.55 mg Apoaequorin / capsule	8Sep2011

Approved by:
(b) (6)
29 Jun 2014

Mark Roeder
Technical and Quality Manager
Quincy Bioscience

000053

8. APPENDIX III

Genetic modification of *E. coli* strain BL21

(Report by Quincy Bioscience)

Pages- 1-6

000054

APPENDIX H: GENETIC MODIFICATIONS TO STRAIN BL21(DE3) BY QB

Quincy Bioscience Genetic modification.

All recombinant methods and cloning techniques were performed according to Sambrook and Russell, Molecular Cloning: A Laboratory Manual 3rd Edition. Quality testing of molecular steps and development of an industrial production strain for the production of apoaequorin strain were performed following ICH guidelines (International Committee for Harmonization), an international regulatory body dedicated to harmonize the best practices for the production of novel biologics by recombinant means for governing bodies and the industry.

Molecular cloning.

An E. coli codon-optimized gene for apoaequorin was produced and inserted into a commercial plasmid vector from Novagen®. This vector possessed a classical T7-lac promoter, a target of the T7polymerase of BL21(DE3). This gene switch is controlled with the use of milk sugar which causes the production of the polymerase in the host strain. The T7 polymerase from the host strain, BL21(DE3), then binds to the T7 promoter of the protein expression gene and apoaequorin is produced by the host cell. This commercial plasmid vector also carries a secretion signal that directs the translation of the gene transcript to the inner membrane of the organisms for further processing, folding of the protein and release into the fermentation medium. The signal sequence is cleaved from off the protein before being releases from the host cell into the fermentation medium. The signal assures the protein sequence is released just before the initial methionine of the apoaequorin protein by type II secretion process.

After initial tests confirmed the sequence of both the gene and the N-terminus of the protein to be correct, the initial cloning step was then digested to release the gene from this plasmid vector. After purification of the DNA, it was then placed into an integration vector to produce the plasmid named pQB4-TS-Kan. The inserted gene included the entire gene from the T7-lac promoter to the T7 terminus. This included the signal sequence.

Using classical molecular cloning techniques the integration vector, carrying the apoaequorin gene, was used to transform the hose cells, BL21(DE3). The cells were then incubated on agar plates for site-specific integration of the protein expression gene. Subsequent screening of recombinant bacterial colonies led to the selection of 6 clones of the successful independent genomic insertions, one of which named QB6#69A was selected for further development and full characterization as the commercial production strain for this project.

Characterization of the production strain.

QB6#69A was further characterized by PCR amplification of the inserted gene to fully characterize the sequence of the gene. Also, the chromosomal flanking regions of the insertion site were verified by the same method to fully demonstrate the site specific localization of the insertion of the apoaequorin gene. No mutations were found of the gene insert. N-terminal sequencing of apoaequorin, purified by SDS–PAGE and blotted onto nylon membrane, demonstrated the correct primary structure of the protein for the first residues: MTSKQYSVKL TSDFDNPRWI GRHKHMFNFL.

Southern blotting of the purified genomic DNA from this clone was blotted on a testing membrane and probed with specific DNA sequences to demonstrate the presence of the expression gene and the absence of plasmid vector DNA. The integration step and design of this integration vector assures the recombination of the apoaequorin gene into the E. coli

chromosome only. This method of blotting also proved that a single, undamaged copy of the expression gene was successfully integrated at the planned site of recombination.

Fermentation development.

Fermentation of the new strain was performed and SDS PAGE and enzyme activity studies (data not shown) were done to demonstrate the protein's molecular weight, the stability of the protein in the culture conditions and the proper folding of the protein (Figure one). This confirmed the molecular weight of the protein being produced in the medium to be approximately 22 kDa and that the protein retained calcium binding activity.

Optimization of the fermentation medium formula through fermentation studies helped to select optimal conditions for growth of the organism and maximization of protein production (Figure two). Kinetic analysis of the fermentation broth by SDS PAGE and luminescent (enzyme) activity was used to demonstrate protein production by the new strain of bacteria. These tests also confirmed that protein accumulation was successful over prolong fermentation times and that the protein did not degrade under these conditions (Figure three).

Cell bank characterization.

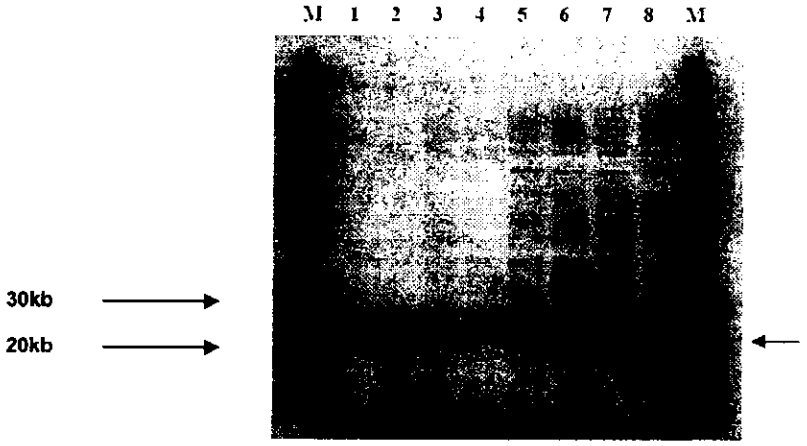
A master cell bank was manufactured from which the purity, identity, lysogenic potential, protein production, N-terminal protein sequencing and sequencing of PCR products of the insert and flanking regions were repeated to qualify the cell bank for future use. This work was done according to the quality assurance guidelines of International Committee for Harmonization: Section Q5B. This full characterization allowed the development of a working cell bank. This cell bank was further characterized for identity, purity, productivity and stability. It is used in the current fermentation process at the contract manufacturer's site.

Genetic analysis of the strain for the presence of the apoaequorin insert can be done by PCR for identity testing.

Conclusion:

Having demonstrated the wealth of knowledge and practical use of this particular strain of the host organism for our process - showing it to be nonpathogenic, mutant in O-antigenic properties and of a lineage to an organisms commensal with the human intestinal system, we have further demonstrated that the genetic transformation of the expression cassette into the strain is stable and inducible as per the intended design.

Figure one: SDS PAGE of protein production by 4 of 6 colonies selected from the development of strain QB6.



M:	Protein size marker
1-4:	Supernatant of the broth harvested at 22 h from the fermentation of integrant clones QB6#44A. #54A. #69A and #82A. respectively.
5-8:	Supernatant of broth harvested at 47 h from the fermentation of integrant clones QB6#44A. #54A. #69A and #82A. respectively.
Arrow:	Expected protein band of the secreted apoaequorin (22.3 kDa)

Figure two: Typical fermentation results for the clone selected, QB6#69A.

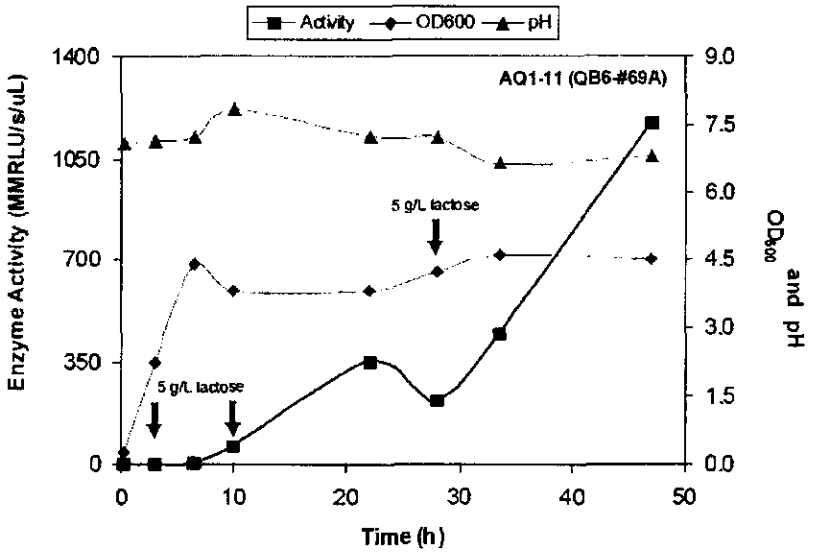
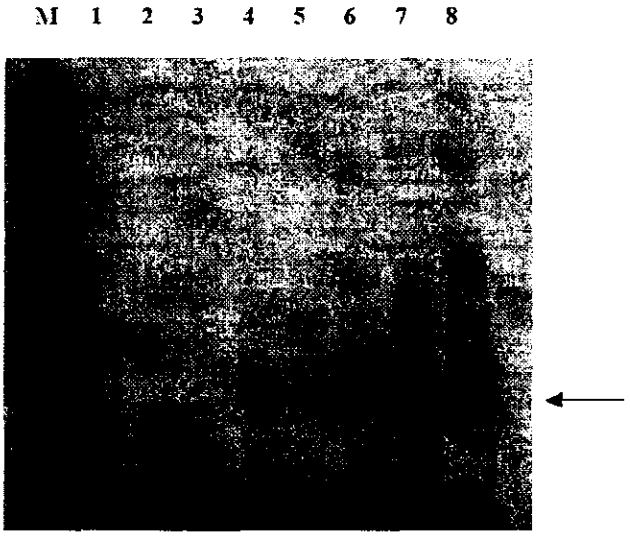


Figure three: SDS PAGE analysis of fermentation at different time points; a kinetic analysis of recombinant protein production.



M:	Protein size marker
1-8:	Supernatant of broth harvested at 0.2, 3.0, 6.5, 10, 22, 28, 33.6 and 47 h, respectively, from 1-liter fermentor AQ1-9.
Arrow:	Expected protein band of the secreted apoaequorin (22.3 kDa)

9. APPENDIX IV

Stability (Digestion) of Apoaequorin Protein in Pepsin

Report by: Dr. Richard Goodman
University of Nebraska

Pages 1-26

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FINAL REPORT

STUDY TITLE

Assessment of the stability of the Apoaequorin protein in pepsin at pH 1.2 and 2.0

AUTHORS

Richard E. Goodman
Afua Ofori-Anti

STUDY COMPLETED ON

8 July 2010

PERFORMING LABORATORY

Goodman Laboratory
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LABORATORY STUDY ID

REG-2010-Pepsin APQ

SUMMARY

The apoaequorin protein used in this study was provided by the study sponsor, Quincy Bioscience of Middleton, WI. The test material was supplied as a liquid protein concentrate on icepacks. The samples were divided in aliquots and frozen at -80°C. The protein was characterized by Quincy Bioscience, demonstrating sequence identity (= P02592), amino acid length (196 aa) and the apparent molecular weight (22.3 kDa). The purity of the protein was calculated to be ~ 94% based on densitometric analysis of a Colloidal blue stained gel with approximately 1.47 µg loaded per lane, analyzed by Kodak Gel Logic 440 with 1 D software.

The apoaequorin protein was diluted in distilled water and then digested in pepsin based on the protocol in Ofori-Anti *et al.* (2008). The pepsin was diluted in simulated gastric fluid (SGF) with the pH adjusted to 1.2 or 2.0 in different experiments. On each assay day the pepsin solution was tested for proteolytic activity by digestion of hemoglobin at the same pH. The mass ratio of pepsin to apoaequorin was approximately 3.6:1, to achieve ~ 10 units of pepsin activity per microgram of test protein. Four digestion assays were performed, two in SGF buffer at pH 1.2 and two in SGF buffer at pH 2.0. Digestions were performed at 37°C under timed conditions. Samples of the digestion mixtures were removed and neutralized at each time point and samples were assessed by colloidal blue stained SDS-PAGE gel band intensities measured by image analysis for the residual apoaequorin. The time to reach 90% digestion of the protein was estimated as the first sample time having less than 10% residual protein compared to diluted non-digested sample protein. The ability of the assay to detect 10% residual protein was determined prior to the assay using serial dilutions of the test protein in a similar SDS-PAGE, colloidal blue stained gel and a 10% undigested control sample was run in each assay SDS-PAGE gel for comparison.

The results of this study demonstrated that the apoaequorin protein was rapidly digested in pepsin at both pH 1.2 and 2.0. The SDS-PAGE colloidal blue gel staining method demonstrated that more than 90% of the protein was digested in less than 30 seconds in all four assays. No residual degradation bands were found to result from digestion of the apoaequorin protein, although a very faint smeared band at approximately 3.5 kDa was evident at 0.5 minutes and nearly gone by 2 minutes. The assays demonstrated good reproducibility. In summary, the apoaequorin protein is rapidly degraded in pepsin at acidic pH. Based on the Codex (2003) guidelines, there would not be a concern of potential allergenicity due to human dietary exposure based on the stability of the protein in pepsin.

Signatures of Report Approval

(b) (6)

8 July 2010

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Date

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Date

Company:	Quincy Bioscience
Company Agent:	Mark Underwood
Title:	President

STATEMENT OF GOOD LABORATORY PRACTICES

This study was not conducted in compliance with Good Laboratory Practice Standards (40 CFR 160, Federal Register, 1989) pursuant to the Federal Insecticide, Fungicide and Rodenticide Act, and subsequent revisions. However, the study was conducted according to accepted scientific methods, and the raw data and study records have been retained.

PRINCIPAL INVESTIGATOR:

(b) (6)

Richard E. Goodman

8 July 2010
Date

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Abbreviations

aa	amino acid
$A_{280\ nm}$	Absorbance of light at a wavelength of 280 nm
APQ	Apoaequorin protein
B	Blank replicate hemoglobin digestion
CFR	Code of Federal Regulations
DC	Control, quenched pepsin, then added test protein as zero time control
dC	Control, quenched pepsin, then added 1/10th the test protein as control
$D_{0.5\text{-}60}$	Digestion samples (apoaequorin plus pepsin) from time 30 seconds to time 60 min.
E_0	Experimental control pepsin without the apoaequorin protein, time 0
E_{60}	Experimental control pepsin without the apoaequorin protein, 60 min.
FIFRA	Federal Insecticide, Fungicide and Rodenticide Act
GLP	Good Laboratory Practice
Hb	Acidified 2% hemoglobin
HRP	Horseradish peroxidase
kDa	kilodalton
LOD	Lower limit of detection
mA	milliampere
mg	milligram
mL	milliliter
mM	millimolar
µg	microgram
µL	microliter
ng	nanogram
NFDM	Nonfat dried milk
P_0	Experimental control apoaequorin protein without pepsin, time 0
P_{60}	Experimental control apoaequorin protein without pepsin, 60 min.
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PVDF	Polyvinylidene difluoride
P/N	Product number, same as catalog number
P1/10	Experimental Control protein apoaequorin 10% sample for gels
R^2	Square of the correlation coefficient
SDS	Sodium dodecyl sulfate
SGF	Simulated gastric fluid (without pepsin)
SOP	Standard operating procedure
T	Test replicate of hemoglobin digestion
TCA	Trichloroacetic acid
Tris	Tris(hydroxymethyl)aminomethane
vdc	volts, direct current
v/v	solute volume to solution volume
w/v	solute weight to solution volume

1. Introduction

Quincy Bioscience, LLC, Madison, WI, developed a genetically modified bacterium, expressing the apoaequorin protein encoded by a gene isolated from the species *Aequorea victoria*, family Aequoreidae, phylum Cnidaria. Quincy Bioscience commissioned a study to evaluate the stability of the protein in a standardized pepsin digestion assay used in assessing biotech proteins for food safety in order to understand whether the protein might pose a risk of de novo sensitization or allergic elicitation.

The Codex Alimentarius Commission guidelines for assessing the allergenicity of GM plants (2003) recommends assessing the introduced protein for stability in pepsin at acidic pH as an assay to help evaluate whether the introduced protein is likely to either increase the rate of sensitization to the host crop, or increase the likelihood of eliciting an allergic response in food allergic consumers. 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 test method for the assessment was first described by Astwood *et al.* (1996). The assay is not meant to predict whether a given protein will always be digested in the stomach of the human consumer, but does provide a simple *in vitro* correlative assay to evaluate protein digestibility. Investigation of proteins that have been tested 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). Purified porcine pepsin has been used to evaluate the stability of a number of food allergens and non-allergenic proteins in a multi-laboratory study that demonstrated the rigor and reproducibility in nine laboratories (Thomas *et al.*, 2004). Porcine pepsin is an aspartic endopeptidase with broad substrate specificity. Pepsin is optimally active between pH 1.2 and 2.0, but inactive at pH 3.5 and irreversibly denatured at pH 7.0 (Collins and Fine, 1981; Crevieu-Gabriel *et al.*, 1999). The assay was refined further in our laboratory, adding an objective measurement of the extent of digestion, which was not previously defined (Ofori-Anti et al., 2008).

The assay is performed under standard conditions of 10 units of pepsin activity per microgram of test protein. Using a highly pure form of pepsin available at Worthington Biochemical, Corp. (#3319), the mass ratio was approximately 3.6 mg pepsin per mg of test protein. 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). Independent assays were conducted at each pH in this study. The assay is performed at 37°C and samples are removed at specific times and the activity of pepsin is quenched by neutralization with carbonate buffer and Laemmli loading buffer, then heating to more than 70°C (~77°C in this study) for 5 minutes. The timed digestion samples are separated by SDS-PAGE and stained with Coomassie or colloidal blue to evaluate the extent of digestion. A review of the

digestibility assay by Bannon *et al.* (2002), Thomas *et al.* (2004) and Ofori-Anti et al. (2008) indicate that most of the non-allergenic food proteins that have been tested are digested in less than 30 seconds, while many major food allergens are stable, or produce pepsin-stable fragments that are easily detectable from eight to 60 minutes (Thomas et al., 2004; Ofori-Anti et al., 2008).

In order to estimate the residual protein content according to the method of Ofori-Anti et al. (2008), a quality control test to verify the activity of pepsin solution was performed and the detection limit of the protein in the stained gels was evaluated. The results of the activity assay do not exactly duplicate the labeled activity determined by Worthington Biochemical, even though similar procedures were used, but the results were within the acceptance criteria previously established in our laboratory (Ofori-Anti et al., 2008). Evaluation of the limit of detection of the colloidal blue stained apoaequorin protein was tested and found to be lower than 10% of the loaded undigested protein, demonstrating the sensitivity to identify 10% residual intact protein.

2. Materials

2.1 Test Substance

The test substance for this study was the apoaequorin protein, identified by the sponsor as lot # 003P143, Aequorin-2 precursor protein (R02592, from *Aequorea victoria*). The solution had been sterile filtered and was in 25 mM Tris, 25 mM NaCl. The sponsor claims the protein mass is 22.3 kDa, with 196 amino acids. In our 10-20% tris-glycine gels, the primary band migrates at approximately 22 to 23 kDa, based on Precision Plus Molecular Weight Markers (Bio-Rad, #161-0374 and Invitrogen (Novex) Sharp Standard #LC5800). The protein arrived on ice-packs, still cold on 29 April, 2010. Concentration based on Quincy Bioscience was 20 mg/ml. The solution was clear and was distributed in aliquots, which were frozen at -20°C.

2.2 Control Substance

There was no control substance for this study.

2.3 Reference Substance

There was no reference substance for this study.

2.4 Characterization of Test, Control, and Reference Substances

Characterization of identity and concentration was conducted by Quincy Bioscience.

2.5 Critical Analytical Reagents

2.5.1 Pepsin, porcine, Worthington Biochemical Corp., product #3319, lot 38C10377, certified as having 2,750 activity units per mg solid.

2.5.2 SGF: A 35 mM NaCl solution is adjusted in pH to 1.2 or 2.0 as measured with a calibrated pH meter, using 6 N HCl, to a final normality of approximately 0.084 for pH 1.2.

2.5.3 SGF plus pepsin: Add a quantity of powdered porcine pepsin to achieve a final activity of 2,632 units per 1.0 mL of SGF. The activity per mg of pepsin is taken from the lot specific -certificate from Worthington Biochemical Corp.

2.5.4 Hemoglobin Worthington Biochemical Corp., product #2403, lot #38E10515 is used to test protein pepsin activity

2.5.6 Colloidal brilliant blue G-250, Sigma Chemical Co., product #B-2025

2.5.7 Pepsin quenching solution: 200 mM $NaHCO_3$, pH 11

2.5.8 6X reducing Laemmli buffer (with 6% β-mercaptoethanol), Boston Bioproducts, #BP-111R, 5 ml, diluted with 1 ml of 2-mercaptoethanol (BioRad cat. #161-0710) to prepare 5X Laemmli with 21.7% of 2-ME.

2.5.9 Precision Plus molecular weight markers from BIO-RAD (product #161-0374)

2.5.10 Invitrogen (Novex) Sharp prestained protein standard (#LC5800)

2.5.11 Invitrogen (Novex) 10-20% tris-glycine polyacrylamide gels, 1 mm thick, 15 wells (Invitrogen #EC61355BOX)

3.0 Test System

The test system for this study was an *in vitro* digestion model using porcine pepsin in simulated gastric fluid (SGF). Standard Operating Procedures (SOPs) for preparation of the SGF, pepsin activity assay, digestion assay, SDS-PAGE, gel staining and western blotting are on record in the laboratory. The SGF preparation and digestion procedures were based on the methods described by Ofori-Anti *et al.* (2008). The pepsin activity assay was based on the method described by Sigma for determining the activity of pepsin.

The amount of pepsin powder used to prepare SGF was calculated from the specific activity on the product label, which in this study was 2,750 units/mg solid (Worthington Biochemical Corp.). One unit of activity is defined as a change in $A_{280\ nm}$ of 0.001 at 37 °C, measured as trichloroacetic acid (TCA)-soluble products using hemoglobin as the substrate. The amount of pepsin used in the digestion mixture is based on the certified value from Worthington Biochemical Corp. The assay is designed for fixed volumes and a fixed amount of test protein so the amount of pepsin diluted in SGF is adjusted to provide the appropriate ratio of 10 units of pepsin activity per microgram of test protein in the digestion mixture. The appropriate amount of solid pepsin is added to SGF to provide 2,632 units per 1.0 mL of SGF. The pepsin/SGF reaction mixture is preheated to

37°C in a water bath before adding 50 microliters of test protein (5 mg/mL) to 0.95 mL SGF + pepsin, for a total volume of 1.0 mL, providing 10 units per microgram test protein.

At predetermined times (between time zero and 60 minutes) a fixed volume of the digesting protein reaction mixture is withdrawn and added to sample tubes containing neutralization and denaturing reagents, which stop the digestion. Samples are then heated to > 75°C before either running in SDS-PAGE analyses or placing in a −20°C for later analysis. All samples from a single digestion are applied to wells of the same SDS-PAGE gel along with molecular weight markers, undigested test protein equivalent to the initial undigested test protein sample and a 10% test protein sample and pepsin alone (to assess pepsin stainable protein bands). Samples are separated by electrophoresis, fixed, stained with colloidal blue G-250, destained and analyzed on a Kodak Gel Logic Image Station with 1D analytical software. The stability of the protein is defined as the time required to achieve 90% digestion which is estimated based on the shortest time-digested sample with a band intensity equal to, or less than the 10% undigested standard well (C0). Any new bands above approximately 3,000 Da that are generated as intermediate products of digestion would be noted as stable (or partially stable) intermediate proteolytic fragments and would be analyzed in addition to the test protein.

Proteins with more than 10% stainable full-length protein band remaining at 60 minutes are considered stable. Proteins reduced to < 10% stainable band at 5 to 30 minutes are considered of intermediate stability. Proteins reduced to < 10% stainable band by 2 minutes are considered labile (rapidly digested).

3.1 Justification for Selection of the Test System

In vitro digestion models are used widely to assess the digestibility of ingested substances. Previous studies have used this simple, *in vitro* assay to evaluate potential risk of food allergy, and demonstrated that digestibility is an important risk factor for food allergy, which could relate to initial sensitization, or to elicitation once the individual is sensitized (Astwood *et al.*, 1996 and del Val *et al.*, 1999). The FAO/WHO (2001) recommended conducting the pepsin digestion assay at pH 1.2 and pH 2.0. Results of a multi-laboratory test using a number of common stable and digestible proteins demonstrated minor differences in stability between the two pH conditions (Thomas *et al.*, 2004). While some authors have claimed a lack of predictive value for this assay (Fu *et al.*, 2002; Yagami *et al.*, 2000), Bannon *et al.* (2002) reviewed a broad range of published representative pepsin digestion studies and found a strong positive predictive value when comparing the stability of allergenic and non-allergenic dietary proteins. As defined by Codex (2003), this assay is not meant to be a stand-alone determinant in evaluating the potential allergenicity of proteins introduced into GM crops, but

the results are to be judged in a weight of evidence approach by regulators. It is also not clear if the risk is primarily about sensitization or elicitation, or a combination of the two.

3.2 Experimental Controls

Controls in this study are meant to ensure assay reliability and include:
Measurement of the activity of pepsin in SGF.

- 3.2.1 Evaluation of the sensitivity of the staining properties of the test protein from serially diluted samples, in a separate, but similar SDS-PAGE gel.
- 3.2.2 Inclusion of samples of pepsin without test protein at times zero and 60 minutes to determine whether any stainable protein bands observed in digestion samples with test protein are from the test protein, contaminants in pepsin or from pepsin autocatalysis.
- 3.2.3 Inclusion of protein in SGF without pepsin at times zero and 60 minutes to evaluate the effect of acid alone.

3.3 Sample Retention

Samples of test protein and digested samples were numbered to distinguish assay time points and assay replicates by date. Residual samples were stored in a -80 °C freezer and will be discarded approximately six months after the completion of the study.

4.0 Detailed Study Methods

This study evaluated the stability of apoaequorin protein 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. A detailed description of the study is presented here. Laboratory records and protocols are on file in the Goodman laboratory, Dept. of Food Science & Technology, University of Nebraska, Lincoln.

4.1 **Verification of Detection System Specificity and Sensitivity.** A preliminary test was performed to ensure that the apparent molecular weight of the apoaequorin protein was as expected and that the range of detection of the SDS-PAGE system with colloidal blue staining and imaging system were capable of detecting the test protein over the range of the experiment (10% to 100% of the test protein mass). Samples of pure apoaequorin protein were prepared and separated in Invitrogen (Novex) 10-20% tris-glycine SDS-PAGE using 1 x reducing Laemmli buffer. Samples of 4.410 and 0.037 micrograms of protein were applied to appropriate wells and Bio-Rad Precision Plus (#161-0374) MW markers were applied to separate lanes. Following electrophoresis, the gel was fixed and stained with colloidal blue G 250 as recommended by Sigma-Aldrich, for a minimum of 6 hours. The gel was destained for 30 seconds in a solution of

10% acetic acid and 25% methanol, then immediately into 25% methanol for several changes, until the background was clear. Densitometric evaluation of the protein was used to determine correlation coefficient over the range of the samples and to ensure that the equivalent of 10% residual mass of protein used in the pepsin digestion assay was detectable (0.147 µg).

4.2 **Preparation of SGF Plus Pepsin.** The simulated gastric fluid (SGF) reaction buffer is prepared by adding 1.23 mg of NaCl to 600 mL of distilled water. The pH of the solution is adjusted to either pH 1.2 or 2.0 using 6 N HCl and 1N NaOH. The HCl content was approximately 0.084 N, and the salt concentration was approximately 35 mM NaCl. The certified activity of porcine pepsin from Worthington Biochemical was used to calculate the amount of solid pepsin that will be dissolved in 1.52 mL of SGF. The target was 4,000 units of activity. For this lot, the certified value was 2,750 units per mg of pepsin solid material. Based on the Worthington Biochemical analysis, 957 mg of solid was added to 1.0 mL of SGF. After mixing to dissolve the pepsin it is stored at 4°C and must be assayed for activity and used within 24 hours.

4.3 **Pepsin Activity Assay.** Each time SGF plus pepsin is prepared for a digestion assay; the activity of the pepsin and the digestion assay were both completed within 24 hours of each other. The purpose of performing the activity assay is to ensure that the pepsin was active within a pre-defined range around the certified claim of activity by Worthington Biochemical. This product typically has an activity of > 2,500 units per mg of solid material. The activity assay we use is similar, but not identical to that used by Sigma-Aldrich, the company that developed the assay. Our tolerance is +/- 23% of the pepsin manufacturer's certified claim. The SGF plus pepsin is stored at 4°C to 8°C until just before use, and then warmed to 37°C before the addition of the target protein. The procedure is performed as follows:

4.3.1 A solution of 2% acidified hemoglobin (Hb) is prepared by dissolving 0.610 g of hemoglobin (Worthington Biochemical Corp. #2403, lot #38E10515) in 24.4 mL of distilled water, then 6.1 mL of 300 mM HCl was added and mixed carefully.

4.3.2 Three polypropylene screw-top centrifuge tubes were labeled as Hb test (# T1-T3), and 3 were labeled as inactivated-pepsin control blanks (#B1-B3), each received 1.25 mL of 2% acidified Hb and all are preheated to 37°C.

4.3.3 At a timed interval (~ 3 mins.), each of the Hb test (#T1-T3) tubes in turn received 0.25 mL of SGF plus pepsin, is mixed by gentle vortexing and returned to the incubator. As each tube reached 10 mins incubation time, 2.5 mL of 0.3 N trichloroacetic acid (Sigma-Aldrich 6.1 N product T0699, diluted 1:20 with distilled water) was added to stop the reaction, the tube

is mixed briefly and insoluble material (undigested hemoglobin) was removed using a PVDF syringe filter (Fisher Biotech product 09-730-27, 25 mm 0.45 μm).

4.3.4 The inactivated-pepsin control tubes (#B 1-3) were interspersed with the test (#t 1-3) tubes. Control tubes (with 1.25 mL of Hb) received 2.5 mL of 0.3 N trichloroacetic acid to precipitate Hb and prevent pepsin activity, then 0.25 mL of SGF plus pepsin and were mixed. These samples were filtered to remove insoluble material (Hb).

4.3.5 The absorbance at 280 nm is measured on a spectrophotometer. The activity units of pepsin per mL is calculated as the mean net absorbance (mean A280 Hb t1-3 – mean A280 control B1-3) multiplied by a conversion factor of 1,000 to yield units of activity per mg of solid pepsin. The tolerance is the certified Sigma-Aldrich factor +/- 23% (+/- 633) units per mg of solid pepsin.

4.4 **Apoaequorin Digestion.** The stock solution of 20 mg/mL provided by Quincy Bioscience was diluted to 5 mg/mL apoaequorin. Since the purity of apoaequorin was estimated to be 94%, based on stained gel (Figure 1), the concentration of original stock was estimated to be 18.8 mg/mL. A working stock of 5 mg/mL was prepared by adding 250 μL of 18.8 mg/mL with 690 μL of dH_2O, for a total volume of 940 μL, which is equal to 5 mg/mL.

4.4.1 Sample stop tubes preparation. 1.5 mL centrifuge tubes are labeled as P_0, P_{60} (apoaequorin protein only in acidic SGF, time zero and 60 mins); DC (inactivated pepsin plus protein, time 0); $D_{0.5}$, D_2, D_5, D_{10}, D_{20}, D_{30}, D_{60} (digested protein in pepsin at specific times from 0.5 to 60 mins); E0, E60 (active SGF plus pepsin at time 0 and 60 mins) and dC (inactivated pepsin plus protein at $1/10^{th}$ apoaequorin concentration, time 0).

4.4.2 Each sample tube (step 4.4.1) received 35 μL of pepsin quenching solution (carbonate buffer) and 35 μL of 5X Laemmli, reducing buffer.

4.4.3 Diluted apoaequorin protein 1:10 for dC sample tube by adding 10 μL the 5 mg/mL stock apoaequorin to 90 μL of distilled water, then vortexing briefly to yield 0.5 mg/mL.

4.4.4 Inactivated pepsin plus apoaequorin control samples (DC and dC): (added 190 μL of SGF plus pepsin to the tubes containing quenching solution, labled as DC and dC. Heat both tubes at >75°C for 10 mins. Then added 5 μL of 1/10 diluted apoaequorin protein (0.5 mg/mL) to tube dC and 5 μL of 5 mg/mL apoaequorin to tube DC, vortex both tubes and heat at > 75°C for 10 minutes before cooling on ice.

4.4.5 Protein only, no pepsin control tube was labeled as P_{mx} received 20 µL apoaequorin protein (5 mg/mL), plus 0.38 mL SGF (without pepsin) and was mixed by gentle vortexing and placed at 37°C.

4.4.5.1 Immediately 100 µL of the P_{mx} was pipetted into the P_0 tube, mixed by vortexing and heated > 75°C for 10 min, then cooled on ice.

4.4.5.2 After 60 minutes in the 37°C water bath, 100 µL of the P_{mx} contents were added to the P_{60} tube, the contents were mix by vortexing and heat > 75°C for 10 min, and then placed on ice.

4.4.6 A pepsin only tube (no enzyme) was labeled E_{mx} and received20 µL distilled water and 0.38 mL of SGF plus pepsin and was then mixed by vortexing.

4.4.5.1 Immediately 100 µL of the E_{mx} contents were pipette into the E0 tube, mix and heat > 75°C for 5 min.

4.4.5.2 After 60 minutes at 37°C water bath, remove 100 µL into the E_{60} tube, mix and heat > 75°C for 10 min.

4.4.7 Digestion reaction mixture tube, labeled as tube D_{mx} added 50 µL of apoaequorin (5 mg/mL) to 0.95 mL SGF plus pepsin, mixed by gentle vortexing and placed in the 37°C water bath.

4.4.7.1 At the appropriate times, 100 µL of digestion mixture samples were withdrawn and pipetted into the appropriate D_X quenching sample tube (e.g. $D_{0.5}$ at 30 sec., D_2 at 2 min), until all samples are quenched (D_{60} minutes). After adding the 100 µL to each tube, the tubes were vortexed briefly, then heated to> 75°C for 10 min and then stored on ice.

4.4.8 When all reactions were complete, test and control samples were either stored frozen or immediately reheated to ~ 95°C for 5 minutes and separated in SDS-PAGE gels (below).

4.5 **SDS-PAGE Gel.** All samples on any one gel are from a single digestion experiment. Invitrogen-Novex 10-20% tris-glycine gels were used with SDS-PAGE buffer, then fixed and stained as described.

4.5.1 All samples are reheated to ~ 95°C for 5 min, then cooled to room temperature and 10 µL are loaded per well (equals 1.47 µg of starting apoaequorin protein per well).

4.5.2 Four µL of pre-stained Bio-Rad Precision Plus molecular weight marker proteins are loaded in one outer well, pre-stained Invitrogen Sharp Standards marker LC5800 was loaded in the opposite outer well.

4.5.3 Electrophoresis is accomplished at a constant 125 vdc.

4.5.4 Gels for staining were fixed in 7% acetic acid, 40% methanol for one hour then stained for a minimum of 6 hours in colloidal blue G250 as detailed

by Sigma, then destained for one min. in a mixture of 10% acetic acid and 25% methanol, then multiple changes of 25% methanol.

4.6 **Image Analysis.** The destained gels are visualized in a Kodak Gel Logic 440 Image Station under white light transillumination. The image is captured and the image intensity adjusted to optimum background and band intensities. The raw image is saved as an archival file.

4.6.1 The molecular weight of the apoaequorin protein and any resulting degradation band that is not in the pepsin only lane is noted.

4.6.2 A single size analysis box is drawn for optimum differentiation of the apoaequorin protein compared to pepsin. The image is copied and pasted to allow density determinations on each sample well, using a single size box.

4.6.3 Minimum perimeter (box) background subtraction was used to reduce the background with minimum image distortion in calculating the net band intensity.

4.6.4 The 10% control band net intensity (dC) is used as the standard for comparison of all digested samples on a given gel.

4.6.5 The first timed digestion well that has a net, band intensity less than the 10% standard is used as maximum time to achieve 90% digestion.

5.0 RESULTS & DISCUSSION

5.1 **Characteristics of apoaequorin in SDS-PAGE.** The apparent molecular weight of the apoaequorin protein in SDS-PAGE was *ca.* 22 kDa (Figure 1).

5.2 **Limit of Detection.** The stained gel of the dilution series of apoaequorin (Figure 1) demonstrated a clear pattern of reduced intensity of stained bands with each step in the dilution series. The minimum amount of protein that was detected was 0.04 µg. Specific net intensity values from the Gel Logic 440 image station were plotted in a regression (Figure 2) 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.

5.3 **Pepsin Activity.** The certified activity of the lot of pepsin from Worthington Biochemical used in this study was 2,750 units per mg of solid. The activity units of pepsin in the hemoglobin assay are listed in Table 1. It was therefore concluded that the activity was acceptable.

5.4 **Digestion Results.** Representative stained gels of digestion experiments at pH 1.2 (Figure 3 and 5) and pH 2.0 (Figure 4 and 6) demonstrated that the apoaequorin protein was in acid alone for 60 minutes (lane 3), but by 0.5 minutes (lane 5) was digested to below the visible band intensity of the 10% apoaequorin control in quenched pepsin (dC control in lane 14). Thus, the estimated time for 90% digestion was less than 30 seconds in all four digestion experiments. Apoaequorin is therefore considered to be a rapidly digested protein.

6.0 CONCLUSIONS

The results of this study demonstrated that recombinant-produced apoaequorin protein was rapidly digested by more than 90% after incubation in SGF plus pepsin at 37°C, based on colloidal blue staining. Based on this study, the digestion characteristics of apoaequorin are similar to those of common non-allergenic dietary proteins. From these data there is no added concern of safety due to unusual stability.

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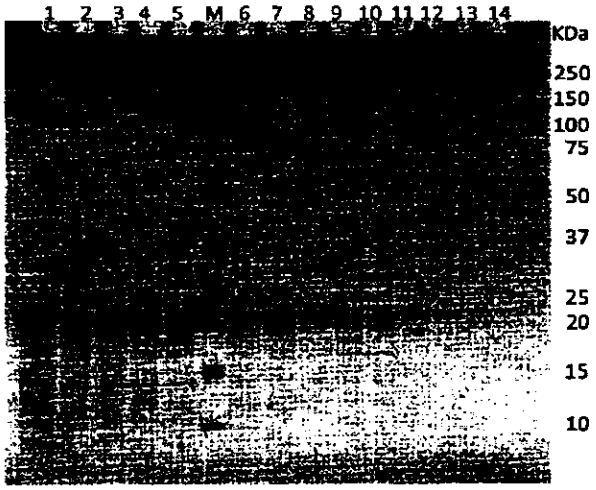

Figure 1. Colloidal Blue Stained SDS-PAGE Gel apoaequorin protein for limit of detection. Samples of a dilution series of the apoaequorin protein was loaded and separated by SDS-PAGE using a 10→20% polyacrylamide tris-glycine gradient gel. A Bio-Rad Precision Plus marker protein was used to estimate MW. Proteins were detected by staining with colloidal brilliant blue G stain.

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

Densitometer Readings of Stained Gel Bands from Figure 1 Sensitivity			
Lane # Figure 1 gel	APQ protein Loaded (µg)	Targeted % Of 1.47 µg	Net Pixel Intensity
6	1.47	100	86380.13
7	1.176	80	82375.22
8	0.882	60	72459.99
9	0.735	50	70165.73
10	0.588	40	58255.71
11	0.294	20	34086.24
12	0.147	10	27114.27

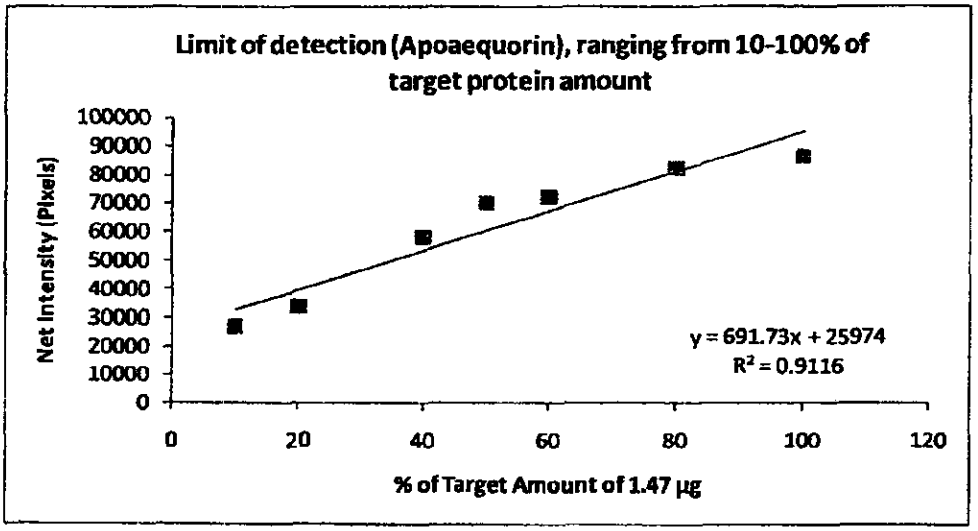

Figure 2. Densitometer readings for the limit of detection of apoaequorin from the colloidal blue stained SDS-PAGE gel (see Figure 1). The Kodak Gel Logic 440 with 1D software was used to make the estimation of stainable protein band. Excel was used to calculate the regression and equivalent protein content based on 100% being equal to 1.47 micrograms per lane.

Table 1. Pepsin activity verification. The activity of pepsin in SGF was verified by digesting hemoglobin using our standard operating procedure, similar to that described by Sigma-Aldrich. Our assay results always differ from either Sigma-Aldrich or from Worthington Biochemical certified lot values, but we have acceptance criteria of target plus or minus 23% from the certified label. If the results are not within the range, the digestion assay is not accepted as valid. For this lot of pepsin, Worthington Biochemical Corp. certified pepsin activity 2,750 +/- 633 units of activity per mg of solid.

Experiment Number	pH of SGF	Pepsin activity units per mg solid pepsin
1	1.2	2,997
2	2.0	3,343
3	1.2	2,176
4	2.0	2,743

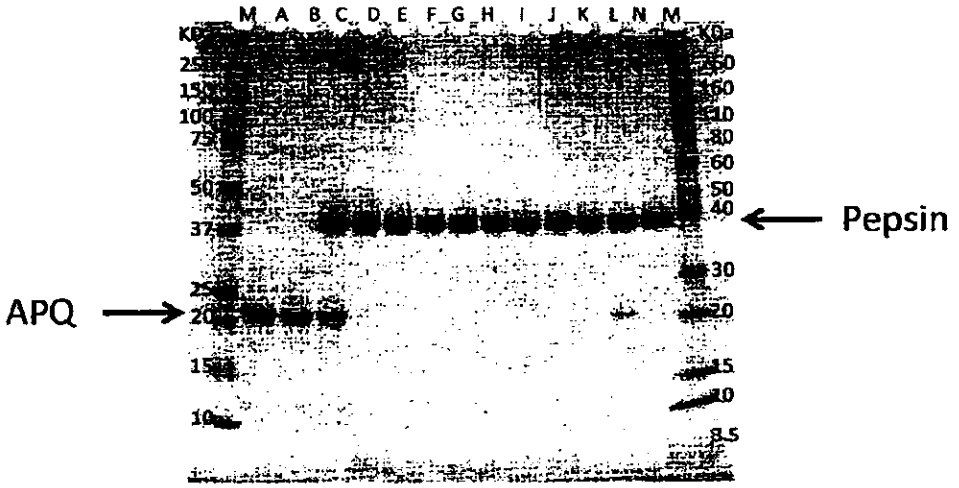

Figure 3. Pepsin Digestion Results on Day 1: for Apoaequorin at pH 1.2, Separated in SDS-PAGE Reducing Gel and Stained with Colloidal Blue. Proteins were digested under standard conditions, and then separated by SDS-PAGE using reducing Laemmli buffer, using a 10→20% polyacrylamide gradient gel. Proteins were detected by staining with colloidal brilliant blue G 250 colloidal stain. Apoaequorin protein was loaded at 1.47μg per lane based on pre-digestion concentration.

Lane	Description	Incubation time
M	Molecular weight marker Bio-Rad	na
A	apoaequorin control without pepsin (P_0)	0 min
B	apoaequorin control without pepsin (P_{60})	60 min
C	apoaequorin in SGF, (DC)	0 min
D	apoaequorin in SGF, ($D_{0.5}$)	0.5 min
E	apoaequorin in SGF, (D_2)	2 min
F	apoaequorin in SGF, (D_5)	5 min
G	apoaequorin in SGF, (D_{10})	10 min
H	apoaequorin in SGF, (D_{20})	20 min
I	apoaequorin in SGF, (D_{30})	30 min
J	apoaequorin in SGF, (D_{60})	60 min
K	Experimental control pepsin (E_0)	0 min
L	Experimental control pepsin (E_{60})	60 min
N	10% apoaequorin with quenched pepsin (dC)	0 min
M	Molecular weight marker Invitrogen	na

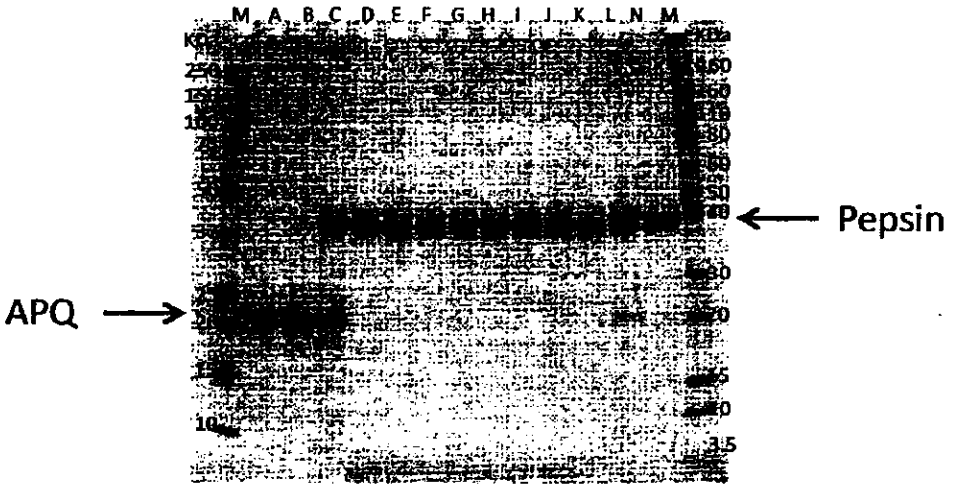

Figure 4. Pepsin Digestion Results on Day 1: for Apoaequorin at pH 2.0, Separated in SDS-PAGE Reducing Gel and Stained with Colloidal Blue. Proteins were digested under standard conditions, and then separated by SDS-PAGE using reducing Laemmli buffer, using a 10→20% polyacrylamide gradient gel. Proteins were detected by staining with colloidal brilliant blue G 250 colloidal stain. Apoaequorin protein was loaded at 1.47μg per lane based on pre-digestion concentration.

Lane	Description	Incubation time	
M	Molecular weight marker Bio-Rad	na	
A	apoaequorin control without pepsin (P_0)	0	min
B	apoaequorin control without pepsin (P_{60})	60	min
C	apoaequorin in SGF, (DC)	0	min
D	apoaequorin in SGF, ($D_{0.5}$)	0.5	min
E	apoaequorin in SGF, (D_2)	2	min
F	apoaequorin in SGF, (D_5)	5	min
G	apoaequorin in SGF, (D_{10})	10	min
H	apoaequorin in SGF, (D_{20})	20	min
I	apoaequorin in SGF, (D_{30})	30	min
J	apoaequorin in SGF, (D_{60})	60	min
K	Experimental control pepsin (E_0)	0	min
L	Experimental control pepsin (E_{60})	60	min
N	10% apoaequorin with quenched pepsin (dC)	0	min
M	Molecular weight marker Invitrogen	na	

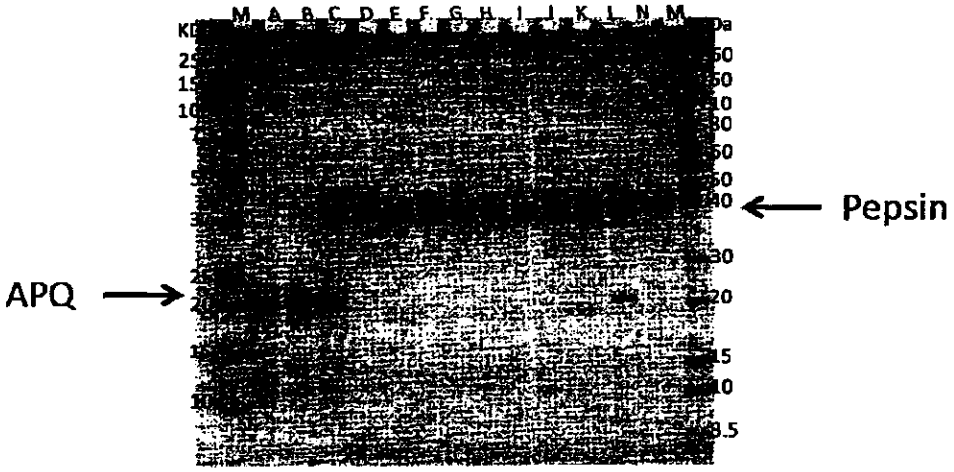

Figure 5. Pepsin Digestion Results on Day 2: for Apoaequorin at pH 1.2, Separated in SDS-PAGE Reducing Gel and Stained with Colloidal Blue. Proteins were digested under standard conditions, and then separated by SDS-PAGE using reducing Laemmli buffer, using a 10→20% polyacrylamide gradient gel. Proteins were detected by staining with colloidal brilliant blue G 250 colloidal stain. Apoaequorin protein was loaded at 1.47μg per lane based on pre-digestion concentration.

Lane	Description	Incubation time	
M	Molecular weight marker Bio-Rad	na	
A	apoaequorin control without pepsin (P_0)	0	min
B	apoaequorin control without pepsin (P_{60})	60	min
C	apoaequorin in SGF, (DC)	0	min
D	apoaequorin in SGF, ($D_{0.5}$)	0.5	min
E	apoaequorin in SGF, (D_2)	2	min
F	apoaequorin in SGF, (D_5)	5	min
G	apoaequorin in SGF, (D_{10})	10	min
H	apoaequorin in SGF, (D_{20})	20	min
I	apoaequorin in SGF, (D_{30})	30	min
J	apoaequorin in SGF, (D_{60})	60	min
K	Experimental control pepsin (E_0)	0	min
L	Experimental control pepsin (E_{60})	60	min
N	10% apoaequorin with quenched pepsin (dC)	0	min
M	Molecular weight marker Invitrogen	na	

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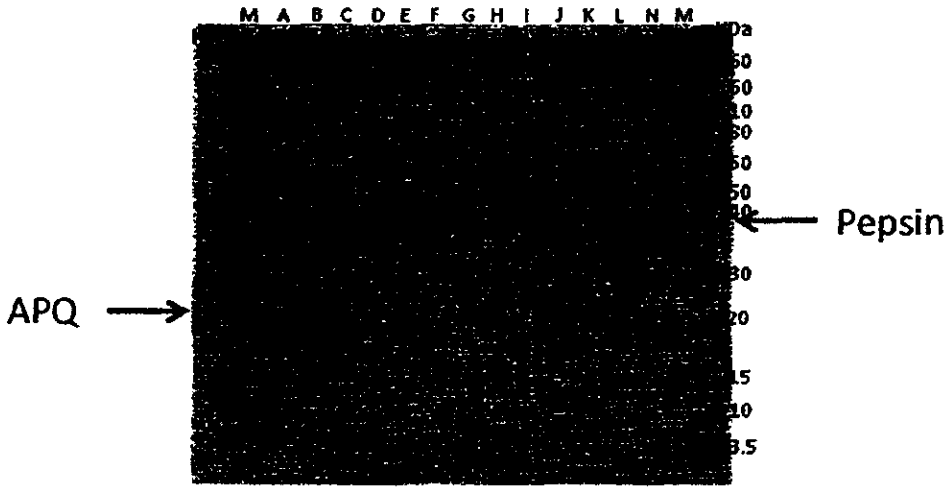

Figure 6. Pepsin Digestion Results on Day 2: for Apoaequorin at pH 2.0, Separated in SDS-PAGE Reducing Gel and Stained with Colloidal Blue. Proteins were digested under standard conditions, and then separated by SDS-PAGE using reducing Laemmli buffer, using a 10→20% polyacrylamide gradient gel. Proteins were detected by staining with colloidal brilliant blue G 250 colloidal stain. Apoaequorin protein was loaded at 1.47μg per lane based on pre-digestion concentration.

Laue	Description	Incubation time	
M	Molecular weight marker Bio-Rad	na	
A	apoaequorin control without pepsin (P_0)	0	mio
B	apoaequorin control without pepsin (P_{60})	60	min
C	apoaequorin in SGF, (DC)	0	min
D	apoaequorin in SGF, ($D_{0.5}$)	0.5	min
E	apoaequorin in SGF, (D_2)	2	min
F	apoaequorin in SGF, (D_5)	5	min
G	apoaequorin in SGF, (D_{10})	10	min
H	apoaequorin in SGF, (D_{20})	20	min
I	apoaequorin in SGF, (D_{30})	30	min
J	apoaequorin in SGF, (D_{60})	60	min
K	Experimental control pepsin (E_0)	0	min
L	Experimental control pepsin (E_{60})	60	min
N	10% apoaequorin with quenched pepsin (dC)	0	min
M	Molecular weight marker Invitrogen	na	

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10. APPENDIX V

Bioinformatic Analysis of Apoaequorin to Assess Potential Allergenic Cross Reactivity

Report by: Dr. Richard Goodman
University of Nebraska

Pages 1-23

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Study Title

Bioinformatic Analysis of Apoaequorin to Assess Potential Allergenic Cross-Reactivity

Authors

Richard E. Goodman

Study Completed On

19 August, 2010

Performing Laboratory

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Laboratory Project ID

Study Number: BIO-01-2010

Study Number: BIO-01-2010

Title: Bioinformatics Analysis of Apoaequorin to Assess Potential Allergenic Cross-Reactivity

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Study Start Date: 19 May, 2010

Study Completion Date: 19 Aug, 2010

Records Retention: All study specific raw data and a copy of the final report will be retained at the Food Allergy Research and Resource Program, University of Nebraska.

Signature of Final Report Approval:

(b) (6)

19 August 2010

Study Author: Richard E. Goodman — Date

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Abbreviations and Definitions

aa	Amino acid
AOL v10	http://www.AllergenOnline.com/ database version 10.0
APQ	Apoaequorin protein origin *Aequorea Victoria*, NCBI accession P02592, GI:113484
Ber e 1	An allergenic 2S albumin from *Bertholletia excelsa* (positive control)
BLASTP	Algorithm used to find local high scoring alignments between a pair of protein sequences (using databases on Entrez)
Entrez NCBI	A public genetic database maintained by the National Center for Biotechnology Information (NCBI) at the National Institutes of Health, Bethesda, MD. Protein entries in the Entrez search and retrieval system are maintained by the NCBI of the National Institutes of Health (U.S.A.)
FARRP	Food Allergy Research and Resource Program, University of Nebraska
FASTA3	Algorithm used to find local high scoring alignments between a pair or protein sequences (using the AllergenOnline database)
GI	A unique identification number assigned by NCBI to each sequence in the database
PubMed	A public information database of scientific journal articles and abstracts maintained by the National Library of Medicine, National Institutes of Health (U.S.A.)
8mer	Exact word search for segments of eight amino acid matches between the query protein and proteins in AllergenOnlin.org

1.0 Summary

The Apoaequorin protein (APQ), originally isolated from the Metazoan, *Aequorea victoria*, was evaluated for potential risks of allergy and allergic cross reactivity using methods typically used for food safety evaluation as prescribed by the Codex Alimentarius Guidelines (2003). The protein sequence is equal to GI:113484 (Inouye et al., 1985) as provided by the study sponsor to represent the recombinant protein expressed in a Genetically Modified Organism developed by the sponsor (Quincy BioScience). The bioinformatics study reported here was designed to compare the protein sequence with those of known allergens in version 10 of the AllergenOnline.org database (AOL v10) maintained by the Food Allergy Research and Resource Program (FARRP) at the University of Nebraska. The purpose of the search is to determine whether the protein is a known allergen (~95% to 100% match to an allergen listed in AllergenOnline.org), or is 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 automatically steps through all possible segments of the protein (cycle 1 compared amino acid 1 to 80, cycle 2 compared amino acid 2 to 81, etc) to identify all possible 80 amino acid segments with >35% identity to any known or putative allergen listed in the AOL v10 database, using the same FASTA3 algoritm. Matches identified by either search would likely be viewed as requiring human serum testing using sera from specifically allergic donors to evaluate potential cross-reactivity (Codex, 2003). The results of both of those bioinformatics searches were negative. Identical searches were also performed using the known allergen Ber e 1, the 2S albumin from Brazil nut as a positive control sequence to demonstrate proper function of the database on the day APQ was evaluated.

Additional information considered in this study included a search of the APQ protein sequence for any 8 contiguous amino acid identity match (8mer) to any known allergen in AOL v10. This step is required by some international regulatory agencies however, it has not been demonstrated to have a substantial predictive value for allergenicity. The results of the 8mer search were also negative.

The APQ sequence was also searched against the non-redundant NCBI dataset using BLASTP with default values for BLOSUM 62 to evaluate homologues of the query protein to better 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. None of those proteins have been identified as allergenic. Homologous proteins in sea anemones (*Nematostella sp.*) had an *E* score of ~ 10^{-8}, while potential homologues in fruit flies were ~ 10^{-4} or larger and identities of < 25%. Additionally, a BLASTP search using the

keyword limit of "allerg*" failed to identity any match to any "keyword" allergen sequence in NCBI with an *E* score smaller than 10^{-3} or a sequence homology of >35% identity.
Finally, references in the PubMed database were searched using the organism name (*Aequorea victoria*) as well as family name (Aequoreidae) and the keyword "allergen" to learn whether there is information to indicate whether the the source organism is a common cause of allergy.The results were negative.

Thus there is no evidence that the Apoaequorin protein is an allergen or would present a possible risk of cross-reactivity to those with known allergies.

2.0 Introduction

The sponsor, Quincy BioSciences, has developed a novel Genetically Modified Organism (GMO), a strain of bacteria expressing the apoaequorin protein from the species *Aequorea victoria*, GI:113484. While there is no intent at this time of using the residual material as a food stuff, there might be the possibility at some time of material from the GMO entering the food supply chain. Thus, the sponsor commissioned a study in our laboratory to to perform a bioinformatics search comparison to known allergens to determine whether there is evidence suggesting potential allergenicity or cross-reactivity (Codex, 2003; Goodman et al, 2008).

Most severe or life-threatening allergic reactions to foods are due to Type I (immediate hypersensitivity) reactions. Such reactions occur when a sufficient number of the individual allergenic proteins, each bound by two separate IgE antibodies, are attached to IgE receptors on the surface of mast cells, stimulating the cells to release histamine and other allergenic mediators. The two binding sites (epitopes) are generally different structures or sequences, unless two identical proteins are covalently linked (e.g. through a disulfide bond). Sensitization to produce the IgE antibodies requires a third allergen-specific recognition sequence within a protein, a CD4+ T-cell epitope, to provide appropriate stimulation to the IgE-producing B cells (Plaut and Zimmerman, 1993). Cross-reactions may be induced in sensitized individuals by a different protein if it contains IgE-binding epitopes that are identical or nearly identical to those of the sensitizing protein. However, cross-reactive ligands usually bind with lower affinity than the immunogen to the antibody and are therefore typically less effective at inducing an immune response (Berzofsky *et al.*, 1993).

The allergenicity assessment includes evaluation of the historical allergenicity of the source of the genes (Codex, 2003). If the source is a major allergen individuals with allergies to the source would be asked to provide serum for IgE binding studies to determine if the protein expressed by the donated gene is an allergen. Extensive literature searches have not identified scientific data implicating *Aequorea victoria* as an allergenic source. Therefore, the focus of the current allergenicity assessment is on the similarity of the three proteins to those of known allergens, based on a bioinformatics analysis.

A bioinformatics search can not identify allergens dc novo. Instead it is meant to identify proteins that are sufficiently similar to a known allergen to suspect that specifically allergic individuals might suffer an allergic reaction because their IgE specific to the allergen would bind strongly to the potentially cross-reactive protein (Goodman et al., 2005). The results of the search are used to identify transgenic proteins that should be tested by specific serum screens, and to identify the specific allergic populations that might be at risk, and should be used as serum donors for specific testing (Codex, 2003).

Screening the amino acid sequences of proteins introduced into plants for partial identity to sequences of known allergens is one of many assessments performed to evaluate product safety (Metcalfe *et al.*, 1996). The extent of sequence similarities between a protein of interest and database sequences of allergens can be assessed using the FASTA sequence alignment tool. While the FASTA and BLASTP programs compare amino acid sequences (*i.e.,* primary protein structure) and provide optimum local sequence alignments. The results may be used to infer similarity of higher order structure (*i.e.,* secondary and tertiary protein structures). Proteins that share a high degree of similarity throughout the entire length are often homologous. Homologous proteins share secondary structure and common three-dimensional folds (Pearson, 1996). Homologous proteins are more likely to share allergenic cross-reactive conformational and linear epitopes than unrelated proteins; however, the degree of similarity between homologues varies widely. It is uncommon to find proteins that are cross-reactive and share less than 50% identity across the full length of the protein sequences, while it is common to find proteins with greater than 70% identity that cause cross-reactions (Aalberse, 2000).

The early bioinformatics comparisons relied on short amino-acid match comparisons designed to identify matches of eight contiguous amino acids (Metcalfe et al., 1996), based on the theoretical consideration that an IgE epitope may be around eight or so amino acids in length. While some IgE epitopes may be as short as five amino acids (Banerjee *et al.*, 1999; Beezhold *et al.*, 1999), the majority of characterized IgE-linear epitopes are eight amino acids or longer (Chatchatee *et al.*, 2001; Reese *et al.*, 1999; Shin *et al.*, 1998). Although many of these reports have demonstrated IgE binding, few have tested the affinity (avidity) of the binding, or the allergic significance of the *in vitro* binding, and it is clear from some reports that high affinity binding requires eight or more amino acids (Banerjee *et al.*, 1999; Rabjohn *et al.*, 1999). In order to induce an allergic reaction, at least two IgE binding epitopes are required on each allergenic protein (or two epitopes that may be identical must be in close enough proximity to cross-link IgE bound to receptors on mast cells or basophils). Because of concerns that some potential IgE epitopes are smaller than eight amino acids, the scientific advisory panel to FAO/WHO (2001) recommended reducing the size of a match to any contiguous six amino acids. However, as demonstrated in a number of bioinformatics papers, searches for six amino acids causes an unacceptably high rate of false positive identities (Hileman et al., 2002; Silvanovich et al., 2006). Further, the positive predictive value of finding identities as short as eight amino acids has not been demonstrated, and the likelihood of false positive matches is predicted to be relatively high, although lower than matches of six amino acids (Silvanovich et al., 2006; Bjorklund et al., 2005). The Codex Alimentarius Commission guidelines (2003) clearly state that the

bioinformatics test and all other tests for the allergenicity assessment should be based on scientifically sound methods. They recommended a FASTA (or BLASTP) search with a threshold of concern of 35% identity as a primary bioinformatics method. Based on information in the scientific literature for sequence identities of clinically demonstrated cross-reactivities, it appears that there are very few cross-reactive pairs of proteins that would not be identified by a scanning window of 80 amino acids with a threshold of 35-45% identity (Goodman and Hefle, 2005; Bjorklund et al., 2005), to compare against a well founded allergen database. Based on the evidence, we are using FASTA alignments to evaluate the transgenic proteins.

In order to maximize the probability of finding any important match of the apoaequorin protein, compared to any allergen, we have used both an overall (full sequence length) FASTA, to find matches of greater than 50% identity as having an increased likelihood of cross-reactivity and we are using an 80 amino acid sliding window to scan the transgenic sequences against the allergen database (AllergenOnline, version 10.0, released January, 20106), using FASTA to search for matches of > 35% identity. In addition, we have used BLASTP to search the current version of NCBI general protein sequences, with the delimiter of "allergen" to identify matches to any sequences that were not in NCBI during the last update of AllergenOnline. In the event such a match was identified, a literature search would be performed to evaluate the evidence that the identified protein was allergenic, including description of symptoms, identification of source organism, expression and identification of the protein and evidence of specific IgE binding. Parameters of the search methods (default settings) and database characteristics are presented in the body of the report. In order to demonstrate that these search methods are reliable, we have also compared the sequence of a known cross-reactive major allergen (Ber e 1) of Brazil nut to database entries using the same criteria and bioinformatics tools.

3.0 Purpose

The purpose of this study was to identify potentially relevant matches in amino acid sequence between the apoaequorin protein and known and putative allergens, which might indicated an elevated risk of allergic reaction to those with specific existing allergies, if they consumed the recombinant protein.

4.0 Methods

4.1 *Protein databases*

4.1.1 AllergenOnline version 10.0 (updated January, 2010). The database is available at http://www.allergenonline.org/. The complete allergen list of 1471 known or putative allergens is shown in Appendix 1 (without sequences). This curated database is maintained by the Food Allergy Research and Resource Program of the University of Nebraska. It includes known and putative allergens that have identified from food, airway, contact and venom allergen sources. Many sequences that are designated as allergen or associated with allergy in the database entry have been filtered out based on

specific criteria. All database entries are linked to sequences in the National Center for Biotechnology Information (NCBI) of the National Institutes of Health (NIH).

4.1.2 NCBI Entrez Protein Database. Protein entries in the Entrez search and retrieval system, maintained by the NCBI of the National Institutes of Health (U.S.A.), and were compiled from the following sources: SwissProt, PIR, PRF, PDB and translations from annotated coding regions in GenBank and RefSeq. The database is potentially updated or modified daily, and therefore the date of sequence searches by BLASTP is relevant to the dataset used in the BLASTP searches. For the present report, all BLASTP searches were run on 19 July, 2010. The database includes all non-redundant GenBank coding sequence translations, the PDB (protein database), SwissProt, PIR and PRF. The total database size on that date was 11,505,486 sequences. The searches with BLASTP were performed using sequences from all organisms, with a filter of "allergen" in the Entrez query. In some cases the sequences are from an "allergenic" organism, and a number are patent sequences. Many of those sequences are not legitimate allergens, or at least lack published proof of allergenicity. In addition, a general unrestricted BLASTP search was run comparing the apoaequeron sequence to all non-redundant entries to identify the most closely related protein sequences.

4.1.3 PubMed. The database of literature references in PubMed were searched for publications that might include reference to allergenicity of the source organism using "*Aequorea victoria*", "Aequoridae" (family) and "Cnidaria" (phylum) combined with "allergen".

4.2 *Sequence database search strategies.*

4.2.1 FASTA3 overall search of AllergenOnline. The potential sequential and inferred structural similarity of the apoaequorin and a positive control allergen, Ber e 1 (2S albumin from *Bertholletia excelsa*) amino acid sequences were compared to all allergens in AllergenOnline version 10.0, by performing a FASTA3 search (Pearson and Lipman, 1988) with these sequences using the default search and scoring criteria of Pearson (2000). The default scoring matrix is BLOSUM 50 (Henikoff and Henikoff, 1992 and 1996). The penalty for each gap inserted into query or searched sequences to obtain optimal alignments is calculated as (q + r*k), where q (−10) is an initial penalty for each independent gap, r (−2) is a penalty for each amino acid position within the gap and k is the number of amino acid positions within the gap (Reese and Pearson, 2002). The default word size (ktup) is two (Pearson, 2000). The FASTA3 version used in these searches was 35.04, dated January 15, 2009, (ftp://ftp.virginia.edu/pub/fasta/). Alignment of regions containing low sequence complexity may lead to irrelevant alignments and are expected to show skewed distributions and should be reanalyzed after removing the low complexity regions (Pearson, 2000). Very small expectation values (*E* values) indicate probable evolutionary homology, and structural similarity. While the *E* value default for FASTA3 is set to 10, a value that does not indicate significant

similarity, distantly related sequences will generally have *E* values less than 0.01, and highly similar sequences that probably represent close homology are more likely to have *E* values less than 1e –7. If the statistical parameters calculated for an alignment between the query protein and any one allergen appear to indicate significant similarity, the percent identity over the length of the intact proteins may be evaluated for possible cross-reactivities in those sensitized to the matched allergen. As discussed by Aalberse (2000), a protein sharing greater than 70% identity over its length, relative to an allergen is likely to be cross-reactive, or share IgE binding. Those that have less than 50% identity are not very likely to be cross-reactive.

4.2.2 FASTA3 of AllergenOnline by 80 aa segments. The identification of relatively short regions of high identity shared by a query sequence and an allergen may indicate similarities that could also share IgE binding, or cross-reactivity. Based on the recommendation of Codex (2003), the FASTA3 algorithm was used to compare all possible contiguous amino acid segments of each of the four test proteins against all sequences listed in AllergenOnline. Every possible contiguous 80-amino acid sequence of each query protein was searched, beginning with amino acids 1-80, then 2-81, 3-82 and so on until the last 80 amino acid segment of each protein was compared with the database on AllergenOnline, using the same FASTA3 algorithm used for the overall-comparison. In this case, only the *E* values and percent identities [(# identical residues / 80 or more amino acids) * 100%)] were evaluated to consider potential cross-reactivity. Alignments of less than 80 amino acids in length were recalculated to normalize the identity to an 80 amino acid score, by increasing the denominator to 80, without altering the numerator. Therefore an alignment with 38 identical amino acids over a length of 40 (=95%), would be recalculated to 47.5%. The reason for the adjustment is that alignments less than 80 amino acids long may have very high identities, and would therefore be more likely to act as a cross-reactive allergen if the matched region represented an IgE epitope, than longer alignments of markedly lower identity scores. When the FASTA3 program inserts gaps in the query sequence to provide optimal alignment, the length of the aligment will exceed 80 amino acids. Rather than "correcting" the alignment identity scores, the same criterion of 35% identity is maintained as per the recommendation of Codex (2003). The rationale by Codex (2003) for recommending that alignments of >35% identity over segments as short as 80 amino acids is that proteins sometimes contain structural motifs that are comprised of sequences much shorter than the intact protein, and that these structural motifs may include a conformational IgE binding epitope. In such a situation, the overall sequence identity for the aligned proteins may be significantly less than 35%, even though a short region could contain an important cross-reactive epitope. This criterion is more conservative than empirical data would suggest is common for cross-reactive proteins (Aalbersee, 2000). It should also help to identify potentially cross-reactive proteins that are not true homologues of an allergen that have significant local identities that might provide an immunological target for IgE antibodies in those with allergies to the matched allergen.

The current algorithm will identify any match containing 29 or more identical aligned, identical amino acids in an overlap of 80 amino acids.

The output of the 80 amino acid FASTA3 search includes a table of each allergen that was matched, and the total number of 80-amino acid matches of greater than 35% identity. An additional file of all alignments is maintained to allow location and further evaluation of any significant match.

4.2.3 Eight-contiguous amino acid search of APQ against AllergenOnline. A word/string search routine was used to identify any eight contiguous amino acid segment of APQ that matches any 8 amino acid segment of any allergen in AllergenOnline. A sliding window word search was performed using the routine available for research purposes on the AllergenOnline.org website.

4.2.4 BLASTP of NCBI Entrez with APQ and "allergen". BLASTP and FASTA3 are unique computer algorithms that provide similar local alignments and results if the appropriate scoring matrices and criteria are used. The BLASTP is available on the NCBI Entrez website (http://www.ncbi.nlm.nih.gov/BLAST/). The current version is BLASTP 2.2.13 (Nov-27-2005). A BLAST search was used comparing each complete query sequence against the entire Entrez Protein database, with a limit option selected to query entries for "allergen", to align only with proteins identified as allergens. The purpose of this BLAST search is to ensure that a significant match with a newly discovered allergenic sequence that has not yet been entered into AllergenOnline.org, is not overlooked. The default conditions for Expectation value (10), word size (3), scoring matrix (BLOSUM62) and gap penalties (-11 existance, plus extension of -1) were used. The "low complexity" filter was turned off, meaning that irrelevant low complexity alignment matches may be identified as significant, so each identified match should be further evaluated to understand the significance. A comparison with the low complexity filter on may also be checked to determine if that is the cause of the match. Evaluation of the *E* value, the length of the alignment and the percent identity of any identified match is necessary to judge the significance of any alignment.

The *E* value (expectation score) calculated by BLASTP is similar to the value calculated by FASTA3. The *E* values and identities calculated for alignments from BLASTP should be judged similarly to those from FASTA3. Since the sequences in AllergenOnline should also be found in the Entrez Protein Database, it is likely that matches of high similarity by FASTA3 using AllergenOnline will also be identified during the BLASTP search. However, sequences of allergens that were published since the compilation of AllergenOnline version 10.0 maybe identified as an alignment by BLASTP, that were not identified by FASTA3. But since most of the "allergen" sequences listed in the Protein database are not true allergens, only identified by sequence similarity, source organism or sequencing by the NIAID, care must be taken to evaluate the data demonstrating

allergenicity for any match identified by BLASTP in NCBI and not identified in AllergenOnline.org.

4.2.5 BLASTP of NCBI Entrez (without keyword limit). A BLASTP was also performed using the sequence of apoaequorin, without using keyword limit, to identify the most closely associated sources of homologous proteins. The purpose was to consider potential evidence of allergenicity or human exposure, without allergy.

4.2.6 PubMed search for publications reporting allergy or allergens from the source organism. PubMed searches were conducted using keywords of the genus and species, family name and phylum name in combination with "allergy" and "allergen".

5.0 Results and Discussion. The complete results for apoaeqourin and the positive allergen control, Ber e 1, will be presented in order.

5.1 Bioinformatics results for apoaequorin. The full-length sequence of apoaequorin (196 amino acids), GI:113484 (Accession number P02592.1) was used to search the AllergenOnline.org database (Figure 1). No signal peptide has been identified for this protein from *Aequorea victoria*, identified by Inouye et al. (1985). Detailed search results and the complete sequence are presented in Appendix 2.

APQ

```
  1 mtskqysvkl tsdfdnprwi grhkhmfnfl dvnhngkisl demvykasdi vinnlgatpe
 61 qakrhkdave affggagmky gvetdwpayi egwkklatde lekyaknept liriwgdalf
121 divdkdqnga itldewkayt kaagiiqsse dceetfrvcd idesgqldvd emtrqhlgfw
181 ytmdpacekl yggavp
```

Figure 1. Apoaequorin (*Aequorea victoria*). The full-length protein (amino acid) sequence of the complete protein is 196 amino acids and was defined by the study sponsors as the protein of interest.

5.1.1 FASTA3 overall search of AllergenOnline version 10.0 with APQ. Results of the FASTA3 search of the apoaequorin protein against AllergenOnline version 10.0 did not identify any significant alignment with an allergen. Scoring results for the four best scoring alignments demonstrate no significant matches with any allergen (see Table 1). The best scoring aligned sequence, a calcium binding protein of olive pollen (*Olea europaea*), followed by a number of fish parvalbumin proteins (Ca+2 binding proteins). While the E scores and identities suggest distant evolutionary divergence from a common ancestor, these similarities do not suggest sufficient identities to warrange laboratory testing using sera from specifically allergic subjects.

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	0.1	33.3	51
131112	*Gadus callarias* codfish	parvalbumin	113	0.29	35.7	56
77799800	*Trachurus japonicus* Japanese mackerel	parvalbumin	107	0.43	34.5	55
14531018	*Theragra chalcogramma* Alaska pollock	parvalbumin	109	0.78	32.7	55

5.1.2 FASTA3 of AllergenOnline by 80 aa segments of APQ. The identity results from FASTA3 alignments of every possible 80 amino acid segment of the apoaequorin protein was compared individually to all sequences in AllergenOnline version 10.0 were all less than 35% over 80 amino acids. There were no matches of greater than 35% identity over 80 amino acids.

5.1.3 Eight-contiguous amino acid match search of AllergenOnline using APQ. The apoaequorin protein sequence was searched against the AllergenOnline.org version 10.0 database using the 8 amino acid word search routine (which has not been demonstrated to be predictive for cross-reactivity, see Goodman et al., 2008). There were no matches of eight amino acid segments between the APQ and any allergen in the database.

5.1.4 BLASTP of NCBI Entrez "allergen" with APQ. The full-length apoaequorin protein was compared to sequences in NCBI-Entrez, which were designated as "allergen" in the NCBI database on 21 July, 2010. There were no alignments with *E* scores at or below "1 x e 10^{-7}", which would indicate probable close homology. Note that the alignments were mostly to identified Ca+2 binding proteins, which are known to have moderately conserved motifs. The level of identity is well below the 50% lower estimate thought by Aalberse (2000) to represent a risk of potential allergic cross-reactivity, and below the conservative 35% identity over 80 or more amino acids used by Codex (2003) as a limit suggesting a need for serum IgE testing.

Table 2. BLASTP of NCBI Entrez "allergen" with APQ. The scoring alignments of ten best scoring matches with *E* scores below 10, to putative allergens in the NCBI Entrez database on 18 August, 2010, were compared to the full-length sequence of apoaequorin, using BLASTP.

Sequence GI #	Organism	Description	Length	*E* score	% Identity	aa Alignment
19386815	*Oryza sativa* Rice	Pollen allergen Jun o 4	185	0.009	31%	73
62249491	*Ambrosia artemisiifolia* Short ragweed	Calcium binding protein	160	0.011	24%	157
14423844	*Olea europaea* Olive	Polcalcin Ole e 3	84	0.033	33%	51
71040671.2	*Arachis hypogaea* Peanut	Calcium binding protein	164	0.14	23%	154
15221781	*Arabidopsis thaliana* Thale cress	Calcium binding pollen allergen	186	0.33	22%	169
14423648	*Olea europaea* Olive	Calcium binding allergen Ole e 8	171	0.35	23%	154
47606030	*Chenopodium album* Lamb's quarters	Polcalcin Che a 3	86	0.52	24%	100
170741818	*Methylobacterium sp.* Methylobacterium	Periplasmic protein thiol—disulphide oxidoreductase	196	0.53	28%	35
223403273	*Litopenaeus vannamei* Pacific white shrimp	Sarcoplasmic calcium-binding protein	193	0.69	23%	114
255583409	*Ricinus communis* Castor bean	Calcium binding protein	154	0.73	29%	55

5.1.5 BLASTP of NCBI Entrez with APQ, without a keyword limit. The full-length apoaequorin protein was compared to sequences in NCBI-Entrez, without limitations. The search was conducted on 19 July, 2010. The alignments follow taxonomic relationships as expected, with isoforms of the protein from *Aequorea victoria E* scores smaller than 1e-100 with identities of between 90 and 100% over their full-lengths. Homologues from other species in the Aequorea genus had *E* scores of from 1e-95 to 8e-98. Less closely related species of hydrozoans have homologues with E scores ranging from 3e-43 to 6e-81 and E scores of proteins of the sea anemone Nematostella vectensis ranginf from 3e-8 to 5e-9. Proteins from highly diverse taxa including the mouse (Mus musculus), round worm (Caenorhabditis elegans), fruitfly (Drosophila sp.) have E scores from 1e-04 to 1e-001, demonstrating that the protein structure and sequence is quite conserved in evolution.

5.1.6 Literature search for allergy to the source of the gene. A PubMed search was conducted to identify any publications that suggest the species (*Aequorea 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 stability of heterologously expressed proteins using the Turnip Mosaic Virus (Chen et al.l, 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 anemonies or treatment of allergic reactions. Only one paper reported identification of the allergenic protein and that (Onizuka et al., 2001) was also identified by search through the three publications identified by "Cnidaria AND allergen", describing allergy to an allergenic protein, of the coral *Dendronephthya nipponica*.

5.1.7 Bioinformatics Summary for APQ. None of the results from the bioinformatics searches with the apoaequorin sequence meet the criteria that might suggest the protein is an allergen or would pose a risk of allergenic cross-reactivity. In addition, the general bioinformatics search demonstrates the protein is highly conserved across a broad range of taxa, with no obvious published indication of allergy associated with homologous proteins. Further, searches of the literature failed to identify a publication demonstrating allergy to *Aequorea victoria* or other family members, but did identify one publication regarding a single protein in a different group within the phylum Cnidaria. There are a number of publications that report allergic or allergic like reactions to various jellyfish, corals and anemonies in this phylum, but a paucity of information regarding the proteins implicated in sensitization. These results suggest that they entire phylum is not a significant cause of allergic reactions in humans.

5.2 Bioinformatics results for Ber e 1. The sequence of the Bertholletia excelsa 2S albumin allergen (Ber e 1) used as a positive allergen control in this report is 146 amino acids long (GI:112754) as shown in Figure 2 (Altenbach et al., 1987).

Ber e 1

```
1   MAKISVAAAA LLVLMALGHA TAFRATVTTT VVEEENQEEC REQMQRQQML SHCRMYMRQQ
61  MEESPYQTMP RRGMEPHMSE CCEQLEGMDE SCRCEGLRMM MMRMQQEEMQ PRGEQMRRMM
121 RLAENIPSRC NLSPMRCPMG GSIAGF
```

Figure 2. Ber e 1 query sequence used as a positive allergen control. The sequence of this protein (GI:112754) has no relationship to APQ, but is used in this report as a positive control to demonstrate bioinformatics results that would be expected with a protein that has been demonstrated to cause some clinical cross-reactivity in some patients, due to structural and sequence similarities.

5.2.1 FASTA3 overall search of AllergenOnline Ber e 1. Results of the FASTA3 search of the 2S albumin protein of Bertholletia excelsa; against AllergenOnline version 10.0 aligned with itself and other known allergenic proteins. The statistical-fit histogram indicated marked deviations from the expected distribution of alignments, with a very narrow and high bell-shaped curve compared to the random distribution expected for most sequences. The reason is that the database contains a high number of 2S albumin proteins. Scoring results for the five highest scoring alignments demonstrate significant matches with four allergens (see Table 7). The identities are above 35% identity over 80 amino acids, which was suggested by Codex (2003) as possibly representing a cross-reactive match. One alignment is markedly above 50% identity, which suggests a strong possibility of cross-reactivity (it is an isoform of the Brazil nut allergen, the others are around 40% identity over the full length, and there is some evidence that a few individuals may have cross-reactivity across these tree nuts, although it is hard to differentiate primary sensitization from cross-reactivity.

Table 3. Overall FASTA3 search of AllergenOnline version 10.0 with Ber e 1. Highest scoring overall alignments of known and putative allergens in AllergenOnline version 10.0, compared to the 2S albumin allergen Ber e 1 using FASTA3.

Sequence GI #	Organism	Description	Length	*E* score	% Identity	aa Alignment
112754	*Bertholletia excela* Brazil nut	2S albumin	146	5.6e-30	100%	146
17713	*Bertholletia excela* Brazil nut	2S albumin large SU	154	2.7e-19	69%	153
1794252	*Juglans regia* English walnut	2S albumin	139	1.5e-9	42%	138
31321942	*Juglans nigra* black walnut	2S albumin	161	2.2e-9	39%	145
5381323	*Sesamum indicum* sesame	2S albumin	148	8.4e-8	39%	148
110349081	*Pistacia vera* pistachio	2S albumin	149	1.7e-6	34%	140
112745	*Helianthus annuus* sunflower	2S albumin	141	1.9e-6	36%	149
24473800	*Anacardium occidentale* cashew	2S albumin	138	4e-6	31%	145
112762	*Ricinus communis* jackbean	2S albumin	258	2.1e-5	39%	104
13183175	*Sesamum indicum* sesame	2S albumin	153	4.4e-5	35%	147
209165427	*Sesamum indicum* sesame	2S albumin	153	5.5e-5	35%	147
17907758	*Fagopyrum esculentum* buckwheal	2S albumin	133	1.9e-4	31%	116
144228127	*Fagopyrum tataricum* Tatary buckwheal	2S albumin	133	1.9e-4	31%	116
26985163	*Brassica napus* rape	2S albumin	109	1.3e-3	31%	107
17225991	*Arachis hypogaea* peanut	2S albumin	144	3.7e-3	28%	142
75114094	*Arachis hypogaea* peanut	2S albumin	144	3.7e-3	28%	146
75107016	*Brassica napus* rape	2S albumin	125	0.01	25%	115
15418705	*Arachis hypogaea* peanut	2S albumin	156	0.018	27%	160
224747150	*Arachis hypogaea* peanut	2S albumin	158	0.018	26%	157
83416591	*Fagopyrum esculentum* buckwheat	2S albumin	149	0.027	25%	150

5.2.2 FASTA3 of AllergenOnline by 80 aa segments with Ber e 1. The identity scores from FASTA3 alignments of every possible 80 amino acid segment of Ber e 1, compared individually to all sequences in AllergenOnline version 6.0 showed 9 proteins (including two Brazil nut entries) had matches of greater than 35% over 80 amino acids. Although there are a few reports of cross-reactivity, or at least co-sensitization of some individuals

with Brazil nut and walnut, sesame seed and peanut, there is not yet any direct published evidence that the 2S albumins are cross-reactive across these species. Based on the identity matches walnut, pecan and sesame seeds are the most likely candidates for cross-reactivity, which could be tested using sera from individuals with clinical reactivity to those species.

Table 4. FASTA3 search with Ber e 1 using a sliding 80mer search of AllergenOnline version 10. Highest scoring overall alignments of known and putative allergens in AllergenOnline version 10.0, compared to the 2S albumin allergen Ber e 1 using FASTA3.

Sequence GI #	Organism	Description	Highest scoring 80mer (%)	# 80mers over 35%	Overall % Identity
112754	*Bertholletia excela* Brazil nut	2S albumin	100%	67	100%
17713	*Bertholletia axcela* Brazil nut	2S albumin large SU	73%	67	69%
1794252	*Juglans regia* English walnut	2S albumin	49%	59	42%
31321942	*Juglans nigra* black walnut	2S albumin	45%	59	39%
5381323	*Sesamum indicum* sesame	2S albumin	44%	67	39%
112762	*Ricinus communis* jackbean	2S albumin	39%	29	39%
112745	*Helianthus annuus* sunflower	2S albumin	39%	40	36
13183175	*Sesamum indicum* sesame	2S albumin	39%	22	35%
209165427	*Sesamum indicum* sesame	2S albumin	39%	22	35%
144228127	*Fagopyrum tataricum* tartary buckwheat	2S alubmin	37%	4	31%

5.2.3 Eight-contiguous amino acid match search using Ber e 1 and AllergenOnline.org version 10. The Brazil nut 2 S albumin, GI:112754 was tested by the eight contiguous amino acid word matching routine to all sequences in AllergenOnline.org. The results identified all possible 8 amino acid segments (139 total) matching itself, GI:112754. In addition, there were five eight amino acid word matches to the homologous Brazil nut protein, GI17713, a second 2S albumin like protein in Brazil nuts identified by Gander et al. (1991). However, the use of short amino acid

search routines has not been demonstrated to be predictive of potential cross-reactivity (Hileman et al., 2002; Silvanovich et all., 2007; Goodman et al., 2008).

5.2.4 BLASTP of NCBI Entrez "allergen" with Ber e 1. The sequence of the 2S albumin from Brazil nut was compared to sequences in NCBI-Entrez, that were designated as "allergen" in the NCBI database.

Table 5. BLASTP search of NCBI Entrez "allergen" sequences with Ber e 1. A number of significanct alignments were identified between the 2S albumin of Brazil nut and other allergens. The 10 best scoring alignments are shown, with significantly low E scores and modest overall sequence identities. There are limited studies demonstrating some in vitro IgE binding is shared among some of these proteins, but the level of biological cross-reactivity is not fully understood.

Sequence Accession #	Organism	Description	Length	*E* score	% Identity	aa Alignment
112754	*Bertolletia excelsa* Brazil nut	2S albumin	146	5e-80	100%	146
AAO32314.1	*Carta illinoisis* pecan	2S albumin	143	1e-22	40%	145
AAB41308.1	*Juglans regia* English walnut	2S albumin	139	9e-20	40%	138
AAM54365.1	*Juglans nigra* black walnut	2S albumin	161	3e-17	39%	127
ACO56333.1	*Corylus avellana* hazelnut	2S albumin	147	5e-17	45%	124
ABG73108.1	*Pistacia vera* pistachio	2S albumin	149	1e-14	32%	140
AAL91665.1	*Anacardium occidentele* cashew	2S albumin	138	2e-14	30%	136
PP01089	*Ricinus communis* Castor bean	2S albumin	256	3e-09	35%	104
AB093594.1	*Fagpyrum tataricum* Tartary buckwheat	2S albumin precursor	133	5e-07	30%	139
NP_194447.1	*Arabidopsis thaliana* Mouse earcrest	2S albumin	166	6e-07	24%	155

5.2.5. Bioinformatics Summary for Ber e 1. A number of significant alignments were identified by all four methods (FASTA3 full-alignment with AllergenOnline, sliding 80 amino acid window FASTA3 search of AllergenOnline, sliding 8 amino acid word match with AllergenOnline and BLASTP with keyword limit of "allergen" with NCBI non-redundant database, using the published sequence of Ber e 1. These demonstrate the proper performance of the bioinformatics searches that were used to evaluate APQ.

6.0 Conclusions

Bioinformatics analyses were performed on the APQ protein under development by the study sponsor (Quincy BioScience) to produce a recombinant bacteria. Results from the bioinformatics search methods used to evaluate the sequence did not identify any significant matches to any allergenic protein. This demonstrates that APQ is not expected to be 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. The AllergenOnline version 10.0 list of 1471 known or putative allergens is presented in Appendix 1. The results of these tests will be maintained as archived records at FARRP, the Department of Food Science & Technology, the University of Nebraska, Lincoln.

7.0 References

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Appendix 1
AllergenOnline.org Version 10.0
Complete database entries.
www.allergenonline.org

Pages 24-288 Available Upon Request

11. APPENDIX VI

REVIEW OF REPORTS OF SERIOUS ADVERSE EFFECTS IN USERS OF PREVAGEN

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REVIEW OF REPORTS OF SERIOUS ADVERSE EFFECTS IN USERS OF PREVAGEN

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I. Introduction

Quincy Bioscience markets a dietary supplement under the brand name Prevagen®. The main ingredient in Prevagen® is apoaequorin, a protein comprised of 196 amino acids.

This report deals with adverse effects (AE) of Prevagen reported to the company. Quincy has a rigorous process for capturing and analyzing AEs. This is detailed in the document "Process for Capture and Analysis of Adverse Events Reported to Quincy Bioscience". Initially, the Customer Service Representative determines the severity of the AE. If the AE appears to be a serious AE (SAE) the reporter is transferred to either the Product Complaint Coordinator or Adverse Event Management. Two subsequent levels of review are followed to determine if the AE is a SAE.

Approximately 2,200 AEs have been reported. Most were not serious; for example, headache, dizziness and nausea were the most common. In reviewing the AEs reported, I studied Quincy's investigation and classification process to determine whether AEs are being adequately classified as non-serious or serious. I also reviewed a sampling of AEs which after investigation were classified as non-serious by Quincy. For example, in July of 2013, a customer called Quincy and reported that her systolic blood pressure spiked to 171. The customer reported always having high blood pressure and indicated her "normal" readings are 150/75. The customer also reported that this was an isolated event and not an ongoing issue after consuming Prevagen since September 2012. No medical intervention was reported during the complaint

investigation. Therefore, in my opinion, this AE was properly classified as non-serious by Quincy. In another instance, a customer reported an instance of low blood pressure. The customer had been taking Prevagen for almost two years. The customer stated that her husband "had mixed up some other medications that she takes for diabetes." Based on the information received from the customer after the investigation, Quincy classified this as a non-serious adverse event. Again, in my opinion, this AE was adequately classified as non-serious. From a review of samples of AEs and the AE summary chart, there is no alarming pattern of SAEs which would indicate a safety concern.

Between 2010 and 2013, Quincy recorded 26 instances of SAEs which were reported to the Food and Drug Administration, a mandatory requirement regardless of a link between the product and the SAE reported. Quincy Bioscience requested a detailed review of the 26 SAE reports. Five reports were made in 2010, ten in 2011, four in 2012 and seven in 2013. These are summarized in Table 1 from Quincy. I have reviewed the Complaint Investigation Forms of these reports and my opinions are provided in this report.

II. Adverse Reports

The information in the Complaint Investigation Forms is organized according to year and number assigned in Table 1.

A. 2010

1) A 7 year old male was reported by his father to have experienced seizures different from his normal seizures. History of Prevagen use 1-3 weeks, one 10 mg capsule per day. Child is epileptic and medications were being changed at the time of seizures.

   Event most likely due to pre-existing condition and medication change.

2) An 82 year old female was reported by her daughter to have experienced a seizure. Event occurred after taking one Prevagen 10 mg capsule per day for one week. No previous history of seizures. Individual takes neuroactive medications.

   Cause of event unclear, but could be due to age and medications.

3) An 80 year old female reported an episode of dizziness and falling. Began taking 10 mg capsules of Prevagen 4 – 5 weeks before event. Individual has Parkinson's disease.

   Event likely related to age and pre-existing condition.

4) A 73 year old male was reported by his wife to be experiencing irregular heart rate and dizziness for 1.5 months. Reported using Prevagen 10 mg capsules for 5 months. Condition started after 3-5 months of use. Individual on several medications.

Event likely related to age and pre-existing condition.

5) A 65 year old female reported experiencing a "mini-stroke". Treatment was not sought. History of Prevagen use not available. Individual ordered 3 month and 6 month supplies after event. Relevant medical history not available.

Nature of event unclear, but did not recur with continued use.

B. <u>2011</u>

6) A female of unknown age (old enough to have an adult daughter) reported an increase in frequency of seizures. Individual had product for approximately 6 months and was reported to take one 10 mg capsule per day. Individual has a history of seizures and is under care of a neurologist.

Event likely due to pre-existing condition.

7) A male of unknown age was reported by his daughter to have experienced worsening of chronic inflammatory demyelinating polyneuropathy which the individual had for 10 years. Individual took one 10 mg capsule per day for unspecified duration. Individual

was undergoing radiation therapy for brain tumor at the time of event.

Event likely represents natural progression of condition.

8) A female of approximately 55 years of age was reported by a friend to have experienced a seizure with falling. Individual took one 10 mg capsule every other day. Approximately 30 years previously, the individual had a concussion followed by persistent memory loss and falling almost daily.

Event likely due to pre-existing condition.

9) An 81 year old male was reported by his daughter to have experienced a stroke. Reported Prevagen use of 40 mg capsules, one per day, for about 2 weeks. Individual had risk factors for stroke.

Event likely due to pre-existing condition.

10) A male of unknown age was reported by his wife to have experienced a seizure. Event occurred after taking two 10 mg Prevagen capsules for 2 days. Individual had a stroke 10 years earlier.

Event likely due to pre-existing condition.

11) An 81 year old male was reported by his wife to have a brain tumor. Took 10 mg capsules of Prevagen, but pattern of use not given. Individual has history of strokes.

Event not related to Prevagen use.

12) A 67 year old male was reported by his wife to have experienced a myocardial infarct. Individual took one 20 mg capsule per day for approximately 9 months. Individual had coronary artery disease treated by bypass surgery.

Event not related to Prevagen use.

14) A 52 year old female reported experiencing hypotension. Individual took one 40 mg capsule per day for unspecified duration. Individual has multiple sclerosis and hypotension.

Event likely due to pre-existing condition.

15) A 54 year old female reported a benign mammary gland neoplasm. Individual took one 40 mg capsule per day in a clinical trial.

Event not related to Prevagen use.

19) A 70 year old male had a stroke. Individual participated in Madison Memory trial in 2010. Individual took Prevagen 10 mg capsules for unspecified duration. Individual was informed that he had atrial fibrillation.

Event likely due to pre-existing condition.

C. **2012**

13) A 58 year old male was reported by his wife to have experienced a seizure. Individual took six 20 mg capsules (one per day) while in Memory case study. Individual has history of brain tumor and seizures.

Event due to pre-existing condition.

16) A male of unknown age reported experiencing hypertension. Prevagen 10 mg capsules taken for 1 week. History of pre-existing hypertension.

Event likely due to pre-existing condition.

17) A 43 year old female reported two relapses of multiple sclerosis. Used Prevagen 20 mg capsules for 3 months, 40 mg for 9 months, and 60 mg for 2 months.

History of multiple sclerosis for greater than 8 years.

18) A 90 year old female was reported by her son to have died. History of Prevagen use vague. Individual had history of cardiovascular disease.

Event likely due to pre-existing condition.

D. <u>2013</u>

20) An 80 year old female reported passing out after consuming Prevagen. The history of Prevagen use was not provided. The patient was taking 6 medications.

Event due to other factors.

21) An 80 year old female was reported by her daughter to have been admitted to a hospital for hypercalcemia. Prevagen 20 mg capsules reported to be used for 20 days. "Symptoms" of hypercalcemia preceded Prevagen use.

Event not due to Prevagen.

22) An 85 year old female was reported by her husband to have experienced chest pain. Individual was taking two 10 mg Prevagen capsules daily for 1 month. Individual was found to have coronary artery disease treated by angioplasty.

Event likely related to pre-existing condition.

23) An 85 year old female reported experiencing nausea, vomiting and flatulence. Individual was taking Prevagen 10 mg capsules for about one month prior to event and resumed after event. Individual reported history of colitis.

Event not likely due to Prevagen.

24) An 85 year old male reported experiencing a slight stroke. Individual was taking Prevagen 20 mg capsules. Individual was taking medications for cardiovascular conditions.

Event likely related to pre-existing condition.

25) An 85 year old female was reported by her daughter to have experienced chest pain, irregular heart rate and elevated blood pressure. Individual was taking Prevagen 10 mg capsules. Event occurred 3 weeks after receiving most recent order. History of elevated blood pressure.

Event likely related to pre-existing condition.

26) An 86 year old female was reported by her daughter to have had a stroke. Individual was taking Prevagen 10 mg capsules twice a day intermittently for an unspecified duration. Individual has history of heart palpitations.

Event likely related to pre-existing condition.

III. Conclusions:

Apoaequorin, the main ingredient in Prevagen®, is a protein which is presumably broken down in the gastro-intestinal tract to amino acids. There is nothing in its structure or preclinical testing to suggest any potential toxicities.

The events reported were almost all anecdotal and mainly neurologic or cardiovascular. They show no commonality in presentation or duration of exposure preceeding event.

Some events (e.g. neoplasia) are not attributable to Prevagen because duration of use too short to lead to event.

Most events were associated with pre-exisiting chronic conditions and are attributable to those conditions..

No increase in reports has occurred in spite of accrual of users which makes causality of events by Prevagen unlikely.

12. APPENDIX VII

REVIEW OF REPORTS OF SERIOUS ADVERSE EVENTS IN USERS OF PREVAGEN

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REVIEW OF REPORTS OF SERIOUS ADVERSE EVENTS IN USERS OF PREVAGEN

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May 30, 2014

PRODUCT

Prevagen contains apoaequorin, which is a protein composed of 196 amino acids. The chemical structure is known and contains 20 different amino acids. It is sold as a brain health supplement to improve memory. The protein was originally identified in certain species of jelly fish. The protein is now made via recombinant DNA in E coli by Quincy BioScience. It has been tested in rats for toxicity, with no reported ill effects.

BACKGROUND

Some 2200 adverse events (AEs) have been reported to the company. Some adverse events reported included chest pain, seizures, tremors, fainting, dizziness, nausea, headaches, and allergic reactions such as itching or skin rashes. These reports were categorized as either "serious" or "non-serious" adverse events. At the request of Quincy, I have reviewed 26 instances of serious adverse events (SAEs) and a representative sampling of non-serious adverse events. In my opinion, Quincy's classification of "serious" or "not-serious" bends on the side of calling more events serious than really are. In reviewing the over-all data, it is my opinion that the product Prevagen is safe. I found no pattern of events that led me to think there was a particular safety problem with the product.

SUMMARY OF SERIOUS ADVERSE EVENTS

Quincy reported 26 SAEs to the FDA between 2010 and 2013. I have reviewed these below.

1. Pt#40-83965; MFR# 40-83965 (2010)

7 yr old boy who was an epileptic. The report is that he experienced seizures different from usual. He was on multiple medications including Keppra, Banzel, Lamictal, Depakote. His anti-epileptic regimen was being changed around with differing anti-epileptic medications. He has taken Prevagen for 1-3 weeks, one 10 mg capsule/day.

Impression: AE is most likely due to his long history of difficult epileptic seizures and changes of medications and not to the Prevagen.

This 82 year old woman experienced a seizure. She gave a history of lung cancer and is on Zoloft, Aricept, and also sleeping pills.

Impression: Not likely this was related to Prevagen.

3. Pt# MS-84421; QBSAER-0001 (2010)

80 year old woman who fell and fractured her hip. Question whether dizziness, poor depth perception, or loss of consciousness could have caused this fall. Patient has Parkinson's Disease and is on medications for this and also for hypertension, arthritis, and colitis. On 10 mg Prevagen for a few weeks.

Impression: She has a number of chronic pre-existing -conditions. Not likely that this was caused by Prevagen.

4. Pt#10-77441; MFR# QBSAER-0001 (2010)

73 year old man with arrhythmias and dizziness for 1.5 months; both bradycardia and tachycardia described; given Mixelitine to slow heart rate; also takes Lyrica and Lunesta; On Prevagen 10 mg for 5 months.

Impression: A number of chronic pre-existing conditions. No evidence that taking Prevagen was related to heart condition.

5. Pt#40-1070492; MFR# 4070492 QA-705-A v4 (2010)

65 year old woman reported having a "mini stroke". Contacted afterward and she said she was fine and continuing to take Prevagen. This has been classified by Quincy as an SAE but I doubt it was. When and how much Prevagen she was taking not available in records.

Impression: not an SAE. No evidence that Prevagen caused this problem.

6. Pt#40-103221; MFR 40-103221 (2010)

A woman reporting more frequent seizures. Patient has a history of seizures. No intervention for present complaint. On Prevagen one 10 mg/day.

Impression: She has a pre-existing chronic condition. Unlikely Prevagen is responsible for complaint.

7. Pt#40-98996; MFR40-98996 (2011)

Patient complained of a flare-up of chronic inflammatory demyelinating polyneuropathy. Essentially "paralyzed". Patient also receiving radiation therapy for brain tumor. Doctor believes the symptoms are progression of his disease. Patient on one 10 mg capsule Prevagen.

Impression: Chronic severe pre-existing conditions. Unlikely Prevagen caused the problems.

8. Pt#40-0109704; QBSAER-00002 (2011)

55 yr old woman who had a seizure and fell down. History of motorcycle accident with concussion 30 years ago. 6 month history of almost daily falling. Patient on Elavil, pain killers, and a variety of herbs and supplements. No other history available. Took one 10 mg Prevagen every other day.

Impression: too little information to come to any conclusion, but it seems unrelated to Prevagen.9. Pt#10-109597; MFR# CN-110038 (2011)

81 yr old patient suffered a stroke and hospitalized. Previous history of risk factors including diabetes, hypertension, hyperlipidemia and "small vessel disease of the brain"; headaches, trouble talking, trouble walking; had dementia and diabetes and hypertension; on many medications: janumet, lisinopril, synthroid, methotrexate, plavix, filate, aspirin, potassium, B12, flomax, DHEA, vit D3, cranberry extract; radiation implants in prostate; one 40 mg cap/day of Prevagen.

Impression: no evidence that taking Prevagen is related to his stroke and other conditions.

10. Pt#10-40-0093338; MFR# CN-110155 (2011)

Patient reported having a seizure. Patient had history of a stroke 10 years prior and open heart surgery 5 months prior. Patient on Coumadin and metoprolol. Seizure occurred after he took two 10 mg capsules for two days.

Impression: Not much information, but preexisting stroke history suggests pre-existing cerebral disease. Unlikely that this is related to Prevagen.

11. Pt#40-107059; MFR CN-110194 (2011)

81 year old male with pre-existing gout. Also had hypertension and diabetes with renal dialysis. Also brain tumor and "large blood clot". Medications included Allopurinol.Colchicine, Insulin, and several other medications for hypertension including diuretics. Taking 10 mg Prevagen/day.

Impression: Pre-existing chronic conditions unlikely related to Prevagen.

12. Pt#40-91240; MFR# CN-113216(2011)

67 yr old man with myocardial infarction who was treated with "quadruple bypass procedure". Patient had risk factors for myocarfdial infarction (hyperlipidemia and hypertension). Patient was on Methotrexate, Lisinopril, Sulfazene, and Diclofenic. He was taking one 20 mg capsule of Prevagen/day.

Impression: man with severe heart disease. No evidence that his problems are related to Prevagen.

13. Pt# 40-111270; MFR# CN-120015 (2012)

58 year old man with glioblastoma multiforme, stage 4. Patient also has history of traumatic head injury and seizures. He was on Cymbalta. Glioblastoma multiforme was removed and he was started on radiation and chemotherapy. He took six 20 mg/day while in a study.

Impression: Pre-existing condition. No evidence his problems are related to Prevagen.

14. Pt# 40-108619; MFR# CN-120045 (2011)

52 year old woman with a period of hypotension reported at 44/12. She has reported previous episodes of hypotension. Also has reported insomnia. On one 40 mg Prevagen/day.

Impression: Not enough information to conclude what went on. No evidence her problem is related to Prevagen.

15. Pt# 40-114820; MFR# CN-120074

54 yr old woman with intraductal papilloma of right breast. Patient has a history of relapsing remitting Multiple Scelrosis, ankylosing spondylitis, memingioma. She takes Copaxane, Propanolol, Zolpidem, Sertraline, Gabapentin, Nitrofurantoin, daily multi vitamin. On Prevagen one 40 mg capsule per day.

Impression: Patient with a number of chronic severe diseases. Problems not likely related to Prevagen.

16. Pt#40-131071; MFR# CN-120193 (2012)

Man with history of hypertension on anti-hypertension medications. Says he had two spikes of BP after taking Prevagen. One went up to 280/180, no data on second one. No sequelae known. Took Prevagen 10 mg/day for a week before first event.

Impression: No evidence that events were related to Prevagen.

17. Pt #40-106887; MFR# 120299 (2012)

43 year old woman with relapsing multiple sclerosis, who is undergoing a relapse manifested by "blurry and double vision, falling, weakness in arms and legs, and pain". On medications Cymbalta, Gabapentin, Percocet, Baclofen, Ambien CR, Nuvigil, Ampyra. She was on 20 mg, 40 mg, and 60 mg over a period of about a year.

Impression: Pre-existing condition which explains her symptoms. Not related to Prevagen.

18. Pt# 40-056558; MFR# CN-120359 (2012)

90 year old woman found dead in her chair. Patient had risk factors for sudden death (myocardial infarction 10 years earlier, coronary artery disease treated with stenting, and cardiac valve problems). She had severe Alzheimer's. High blood pressure and ruptured intracranial aneurysm postulated for her death.

Impression: Pre-existing conditions explain her death. Not related to Prevagen.

19. Pt# CN-130077; MFR# QB-40-085112 (2011)

70 year old man who had a right hemisphere stroke, causing left homonymous hemianopsia. Patient had risk factors for stroke (atrial fibrillation and hyperlipidemia). Patient was on Lipitor, Atenolol, Aspirin. Took 10 mg Prevagen/day; not clear how long.

Impression: atrial fibrillation was most likely the cause of the stroke. No evidence that Prevagen was involved.

20. Pt# 40-106579; MFR# CN-130419 92013)

80 year old woman who "passed out". She was on metoprolol, lisinopril and pradaxa. The fact that she was on Pradaxa suggests she might have atrial fibrillation or other arrhythmia. No other information available.

Impression: Little information for conclusion, but no evidence that Prevagen was involved.

21. Pt#40-172426; MFR# CN-130478 (2013)

80 year old woman with hypercalcemia with symptoms since June 2013; taken to hospital with Calcium of 15. Took Prevagen 20 mg/day from 8/1 to 8/21/2013;

Impression: Not enough information for conclusion as to cause. No evident relation of high calcium with Prevagen.

22. Pt#40-160248; MFR# CN-130491 (2013)

85 yr old woman who had chest pain and had a "heart attack". She was hospitalized and two of her coronary arteries were "cleared out". She had family history of heart disease (father). She had been taking 20 mg/day Prevagen for one month.

Impression: no evidence the heart attack was related to Prevagen. It takes many years for coronary arteries to clog up and she had been taking Prevagen a few months.

23. Pt#40-177606; MFR#CN-130578 (2013)

85 year old woman with colitis, hypertension and heart disease. Sick to stomach and vomiting and was admitted to hospital.. Discharged apparently with the same diagnoses. She had been on 10 mg/day Prevagen.

Impression: Woman with chronic abdominal disease. Problem not likely related to Prevagen.

24. Pt#40-168283: MFR#CN-130533 (2013)

85 year old man experienced a "slight stroke". He was discharged from hospital after one day. He was taking Areda formula, Pravastatin, Isosorbide mononitrate, Doxazocin, Coumadin, Docusate calcium, vit D3, Vit e, Metoprolol, Imipramine. He was also on Prevagen 20 mg/day. No other information available.

Impression: He was taking multiple drugs for multiple chronic conditions. Unlikely Prevagen was related to his event.

25. Pt#40-073139; MFR# CN-130548 (2013)

85 year old with chest pain, irregular pulse, and elevated blood pressure. She has pre-existing hypertension and macular degeneration. Her usual BP is 160/80. She was reported to have 246/226 at hospital. Patient discharged on second day. Apparently there were no sequelae. Had been on 10 mg twice a day Prevagen for 3 weeks.

Impression: Patient with longstanding hypertension. Prevagen unlikely implicated in this SAE.

26. Pt#40-172783; MFR#CN-130530 (2013)

86 year old woman who had stroke. Gave history of heart palpitations. She was taking Prevagen 10 mg twice a day, though intermittently. She was not on other medications. No other information available.

Impression: Very little information. Unlikely that this was caused by Prevagen.

OTHER REPORTS REVIEWED:

Pt#40-121126; MFR# CN-130535 (2013)

Woman complaining of low blood pressure of below 65. Taking Prevagen for 2 years/ Patient also diabetic. Says her husband mixed up her diabetes medications.

Impression: should not be considered an Adverse event.

Pt#40-106579; MFR# CN-139419

90 yr. old woman: took green powder or green tablet (unclear which) and shortly after passed out and was hospitalized. She was subsequently released home with no evident sequelae. She also takes pradaxa (anti-coagulant) and metoprolol (B blocker); also lisinipril, ranitidine, and donepezil;

Impression: No evidence that the problem was caused by the Prevagen. It is not even clear that she was taking Prevagen at the time.

Pt#40-156568; MFR# CN-40-130423

Man reported a rise in blood pressure while taking Prevagen. Also on Crestsor, Niacin, aspirin. He normally reports low blood pressure. He stopped taking Prevagen and is fine.

Impression: I doubt this is an Adverse Event. If so, not related to Prevagen.

#QA-705-B v4

Woman suddenly died; cause unclear; had heart attack in 2002 and had stents put in, then had heart valve problems; taking depocal for mood but stopped it a few days before her death; Was also taking pregnenalone.

Impression: no evidence that death related to Prevagen.

#40-146267

Woman reported that blood pressure rose from 150 systolic to 186; long history of high blood pressure; on Prevagen; blood pressure only spiked and returned to previous readings; continued to take Prevagen; Impression: no evidence that spike in blood pressure due to Prevagen.

Pt#40-172059' MFR# CN-130463 (2013)

Complaint of blood pressure rising to 172/83. Customer has hypertension and is on a number of anti-hypertensive medications. Admits to not being very good about taking her medications. Was on Prevagen.

Impression: no evidence higher blood pressure related to Prevagen.

Pt#40-175477; MFR# CN-130595 (2013)

Woman reported high blood pressure reading: 174/99. She was on anti-hypertension medication. No further problems reported. Taking Prevagen Extra Strength 20 mg.

Impression: Chronic condition. Not related to Prevagen.

Pt#40-179420; MFR# CN-130617

Subject with hypertension and atrial fibrillation. On medication for hypertension and hypercholesterolemia. Took Prevagen for one week before episode of high BP.

Impression: Chronic conditions not related to Prevagen.

Pt#40-176927; MFR# CN-130556 (2013)

Man with diabetes and hypertension who complains of higher blood pressure while taking Prevagen. On Lopressor, Benicar, glucophage, Zocor, and Zetia plus a number of vitamins and minerals. Blood pressure better within a month.

Impression: Chronic conditions not related to Prevagen.

Conclusion:

Impression: Chronic conditions not related to Prevagen.

CONCLUSION:

After reviewing the 26 reports characterized as "serious adverse events" by Quincy and several complaints classified as "non-serious," I can state with a high degree of confidence that Prevagen was likely not the causative agent. Many of the individuals either had pre-existing conditions, some of which were complicated, and others were on several concomitant medications. As discussed above, I found no pattern of events that led me to think there was a particular safety problem with Prevagen.

(b) (6)

Xavier Pi-Sunyer, M.D., M.P.H.

June 16, 2014

13. APPENDIX VIII

Adverse Events Report Review Process and Summary

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Registered Patent Agent

June 10, 2014

Madhu Soni, PhD, FATS
Soni & Associates, Inc.
749 46th Square
Vero Beach, FL 32968

Re: Quincy Bioscience Manufacturing, Inc. – Adverse Event Review

Dear Dr. Soni:

As you know, we represent Quincy Bioscience Manufacturing, Inc. ("Quincy") in regulatory matters. We are writing to provide you with additional information regarding Quincy's investigation of adverse events reported by consumers of the Prevagen® supplement product containing apoaequorin. In addition to its own comprehensive review of the adverse events reported, Quincy has also retained two prominent physicians to analyze the serious and non-serious adverse events. Both physicians concluded that in their opinion, there was no safety concern attributed to use of Prevagen®, and that in all likelihood Prevagen® did not contribute to the adverse events reported.

Quincy's Review of Adverse Events

Quincy has a stringent complaint and adverse event review procedure in place which requires thorough investigation of all complaints reported to Quincy, along with an internal procedure for classifying relevant complaints as either adverse events or serious adverse events (*See* **Exhibit 1** – Quincy Adverse Event Procedure Summary and Accompanying SOPs). Until January 2014, Quincy has received roughly 2,200 reports of adverse events, with only 26 of the reports meeting Quincy's threshold for serious adverse event (*See* **Exhibit 2** – Adverse Event Summary Table).[1] In the interests of being as comprehensive as possible in its review of all complaints, Quincy also submitted a Freedom of Information Act (FOIA) request to FDA to ensure that all serious adverse event reports have been accounted for (*See* **Exhibit 3** – Quincy FOIA Request). The results of the FOIA request returned 26 serious adverse events in FDA's records, a number which matched Quincy's own records. Quincy compared the reports in FDA's files and determined that they were the same ones that Quincy had submitted to FDA. Please note that Quincy has also re-reviewed all reports received against its stringent review and classification policy.

[1] An adverse event is defined as any health-related event associated with the use of a dietary supplement that is adverse (21 U.S.C. 379aa-1(a)(1)). A "serious adverse event" is an adverse event that: (a) results in death, a life-threatening experience, inpatient hospitalization, a persistent or significant disability or incapacity, or a congenital anomaly or birth defect; or (b) requires, based on a reasonable medical judgment, a medical or surgical intervention to prevent an outcome described above (21 U.S.C. 379aa-1(a)(2)).

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Expert Physician Review

Quincy retained the services of two expert physicians to review the 26 serious adverse event reports, as well as a number of non-serious adverse event reports, in order to determine whether causality exists between the apoaequorin ingredient and the adverse events reported. The physicians also reviewed Quincy's adverse event investigation process. Both physicians concluded that Prevagen® was unlikely to have caused the serious adverse events reported (*See* **Exhibits 4-5** – Report of Dr. Williams and Report of Dr. Pi-Sunyer; *see also* **Exhibits 6-7** CV's of Dr. Williams and Dr. Pi-Sunyer). Both physicians ultimately concluded that the Prevagen® containing apoaequorin was likely not the cause of the serious adverse events reported. In many instances, both physicians completely ruled out Prevagen® as the cause of the serious adverse event experienced. It is important to note that subjects in all of the serious adverse event reports either were on concomitant medications or had pre-existing conditions which likely contributed to the serious adverse events reported.

Conclusion

As indicated above, the likelihood that Prevagen® was not the causative agent in non-serious and serious adverse events reported by consumers is further corroborated by the opinions of the two prominent physicians who reviewed the adverse events, as well as Quincy's adverse event investigation and classification procedures.

Please do not hesitate to contact me at 312.327.3325 or Abhishek@AminTalati.com if you have any further questions or concerns.

Sincerely,

(b) (6)

Abhishek K. Gurnani

Exhibit 1

Process For Capture And Analysis Of Adverse Events Reported To Quincy Bioscience

All Adverse Events reported to Quincy Bioscience are captured for analysis. Each and every Adverse Event reported to Quincy Bioscience undergoes a three-step/level review process to determine whether a particular AE is serious as defined in 21 U.S. CODE § 379AA–1 and must be reported on FDA Form 3500A.

The first step in the aforementioned review process is when the Adverse Event is first reported to Quincy Bioscience. Quincy Bioscience typically becomes aware of an Adverse Event by one of three different avenues. Adverse Events are typically reported to Quincy Bioscience either when the customer/reporter calls the number on our product's label or during a routine, outbound customer service calls to customers who have previously purchased product directly from Quincy Bioscience. Occasionally, a customer will return product by shipping it back directly to Quincy Bioscience without any prior contact with the company.

Upon receipt of an Adverse Event, information from the customer/reporter is captured by the Quincy Bioscience Customer Service Representative (CSR) in the Complaint Recipient section of Standard Operating Procedure Form QA-705-B. This form is stored as a Microsoft Word template and the information collected is entered into the Complaint Recipient section of Form QA-705-B.

The following information is requested by the Quincy Bioscience CSR from every customer/reporter of an Adverse Event:

- Customer's identifying information,
- Gender,
- What product does this report involve,
- Where the product was purchased,
- Specific Lot Number,
- What Adverse Effects were experienced by the customer,
- How many capsules was the customer taking daily,
- Has the customer stopped taking the product,
- How long had you been taking this product,
- When did the Adverse Event begin,
- How is the customer feeling at the time of the report,
- Did the customer seek medical attention for this issue,
- Was the customer admitted to a hospital,
- What other, if any, supplements or prescription medications does the customer take,
- Does the customer have any allergies and if yes, what are they,
- What are, if any, the customer's ongoing medical conditions/concerns,
- Has the customer made any changes to their normal routine since beginning to take our product or since the Adverse Event began,
- The customer is asked if there is anything else that they want to tell us.

Depending on the Adverse Event being reported, additional information may be requested to facilitate the determination of the severity of the Adverse Event. For example; for hypertension, the

customer's adverse blood pressure reading(s) and normal systolic and diastolic numbers would be recorded. For a rash, the extent of the rash would be recorded (which areas, percent of area covered by rash, etc.). This information will be used to determine the severity of the Adverse Event and its impact on the customer's activities of daily living (ADL) and instrumental activities of daily living (IADL) as appropriate.

ADL examples: walking, bathing, dressing, toileting, brushing teeth, eating. IADL examples: cooking, driving, using the telephone or a computer, shopping, keeping track of finances, managing medication. IADL tasks support an independent lifestyle.

If the customer/reporter refuses to provide the necessary information or if there are inconsistencies in the information provided, the Quincy Bioscience CSR receiving the Adverse Event Report (AER) will attempt to clarify the confusion. For example, if the customer has listed a number of prescription medications but says that they don't have any ongoing medical conditions/concerns, the CSR will inquire as to why the customer is taking a particular medication.

The Quincy Bioscience CSR will then make an initial determination of the severity of the Adverse Event (Adverse Event vs. Serious Adverse Event) and determine whether return of product will be requested. Every instance of inpatient hospitalization is categorized as a Serious Adverse Event. Emergency room visits without formal hospital inpatient admission may be either an Adverse Event or Serious Adverse Event, depending on the Adverse Event details. All product associated with Serious or potentially Serious Adverse Events will have a request made for its return. Product return is not requested for all Adverse Events. Once all of the information is collected on Form QA-705-B, the form is printed out, signed, and dated.

If the Adverse Event appears to be a Serious Adverse Event, the customer/reporter is transferred to either the Product Complaint Coordinator or Adverse Event Management immediately after the Quincy Bioscience CSR completes the Complaint Recipient section of Form QA-705-B.

The second level of review for an Adverse Event occurs after the Quincy Bioscience CSR has completed the Complaint Recipient section of QA-705-B. The Product Complaint Coordinator reviews Form QA-705-B and verifies that all necessary information has been collected from the customer/reporter on the complaint form.

The Product Complaint Coordinator assigns a Complaint Number and records information in the Complaint Log QA-705-D. The Product Complaint Coordinator reviews the category assigned by the Complaint Recipient. If the Adverse Event is a Serious Adverse Event or a potential Serious Adverse Event, Adverse Event Management is immediately contacted.

The third level of review occurs with Adverse Event Management. All Adverse Events are reviewed by Adverse Event Management. The information on form QA-705-B is reviewed to determine the type and classification of Adverse Event.

Adverse Event Management assigns a severity grade for each Adverse Event based upon the details of the individual complaint. All Adverse Events are graded based upon the information in The National Cancer Institute-Common Terminology Criteria for Adverse Events -Version 4 (CTCAE 4.0).

Any Adverse Event that is graded as a 3 or greater based upon the CTCAE 4.0 will be investigated further by Adverse Event Management. If Adverse Event Management determines that additional information is required from the customer/reporter an investigation will be initiated. The customer/reporter will be contacted and additional information about the customer/reporter and the Adverse Event will be collected as required to complete Form QA-705-A, the Complaint Investigation Form.

In addition to collecting additional information from the customer/reporter, an investigation may include any of the following activities as appropriate: A historical complaint review, internal batch record review, contract facility investigation, returned and/or reserve product testing, evaluation of additional impacted lots, and inventory assessment.

All Serious Adverse Events will be investigated. Any Adverse Event determined to be a valid SAER will be reported to the FDA within 15 working days of the initial receipt of the Adverse Event, as per QA-706.

Upon completion of all investigations and any necessary filings copies of all types of Adverse Event reports are kept in binders in a secure office location.

QUINCY BIOSCIENCE	Standard Operating Procedures # **QA-705 v5**
Replaces Version: v4	Effective Date: **October 9, 2013**
Title: **Complaint Handling and Investigation**	

1. **PURPOSE**
   1.1. The purpose of this document is to provide a procedure describing how complaints associated with a dietary supplement product are documented and then managed.

2. **SCOPE**
   2.1. This document applies to any Quincy Bioscience (QB) employee or additional associates who are involved with documenting and managing reported complaints.

3. **RESPONSIBILITIES**
   3.1. Product Complaint Coordinator: Review complaint form and MAS data for completeness. Conduct initial evaluation of all AEs and MDs. Assign a Product Complaint Number to all complaint forms.
   3.2. Adverse Event Management: Review all reported AEs. Initiate AE investigations and conduct trend analysis of reported AEs. Responsible for review of corrective/preventative action proposed in response to an AE.
   3.3. Manufacturing Defects Coordinator: Review all reported MDs. Initiate MD investigations and conduct trend analysis of reported MDs. Responsible for review of corrective/preventative action proposed in response to an MD.
   3.4. Complaint Recipient: Obtain necessary information from customer regarding the nature of the complaint (dates, details, etc.). Complete the appropriate complaint form. Enter applicable information into MAS.
   3.5. Holding & Distribution (H&D): Ensure that any bottle returned by customer associated with a complaint is routed to appropriate personnel per QA-704.
   3.6. Quality Management: Review and approve completed investigations.

4. **REFERENCES AND DOCUMENTATION**
   4.1. Return Merchandise Authorization (RMA) (QA-704)
   4.2. Return Merchandise Authorization (RMA) Form (QA-704-A)
   4.3. Complaint Investigation Form (QA-705-A)
   4.4. Adverse Event Report (AER) Form (QA-705-B)
   4.5. Manufacturing Defect (MD) Form (QA-705-C)
   4.6. Complaint Log (QA-705-D)
   4.7. Serious Adverse Event Reporting (SAER) (QA-706)
   4.8. National Cancer Institute-Common Terminology Criteria for Adverse Events (CTCAE) version 4

5. **DEFINITIONS**
   5.1. **Dietary Supplement Product (Product)**: Any dietary supplement item sold, distributed, or provided to a customer by Quincy Bioscience.
   5.2. **Complaint**: Any complaint or expression of dissatisfaction with a product. Can be related to either an Adverse Event or a Manufacturing Defect.
   5.3. **Complaint Number**: A unique, sequential number assigned to a customer complaint for identification and tracking purposes. Number format is CN-YYXXXX, where YY is the last two digits of the current year and XXXX is a sequentially assigned number.
   5.4. **Reporter**: Person or institution reporting a complaint (Customer, Doctor, or Hospital personnel, for example).

Issued to: Westfield
QA: (b) (6)
Date: 09OCT13

5.5. **Complaint Recipient**: Any Quincy Bioscience (QB) employee, representative or agent of Quincy Bioscience who receives a report from a customer or responsible reporter of a product complaint associated with a product.

5.6. **Adverse Event (AE):** An undesirable or unexpected effect that occurs after the use of a product. The event may or may not be caused by the product.

5.7. **Adverse Event Report (AER)**: A process for collecting information associated with an AE.

5.8. **Serious Adverse Event (SAE):** Any undesirable or unexpected medical occurrence or effect that occurs after the use of a product. The event may or may not be caused by the product. Examples of SAEs include:

- death
- a life-threatening condition
- inpatient hospitalization
- a persistent or significant disability/incapacity
- an intervention to prevent permanent impairment or damage

5.9. **Manufacturing Defect (MD):** Any complaint or problem with capsules, powder, seals, labels, or packaging.

5.10. **Attachments**: Copies of customer or responsible reporter correspondence, written, or electronic notes regarding verbal communication with customers or responsible reporters, reports, records of investigations, and any associated forms.

5.11. **MAS**: Accounting software.

6. PROCEDURE

6.1. Adverse Events

6.1.1. Receipt of Complaint

6.1.1.1. Upon receipt of an adverse event, the complaint recipient captures the initial information from the reporter by completing the Complaint Recipient section of form QA-705-B.

6.1.1.1.1. A Microsoft Word template of this form will be used to type information into the Complaint Recipient section. The form is not considered official until it is printed out, signed, and dated by the complaint recipient.

6.1.1.2. If the complaint appears to be a Serious Adverse Event, connect the reporter to the Product Complaint Coordinator or AE Management immediately after completing the Complaint Recipient section of form QA-705-B.

6.1.1.3. If both the Product Complaint Coordinator and AE Management are not available, inform the reporter that they will be receiving a follow-up call from AE Management.

6.1.1.4. If the complaint appears to be a Serious Adverse Event, inform the reporter that they will be receiving a pre-paid envelope to return their product.

6.1.1.5. Enter lot number, complaint description, and any other applicable information in a customer memo in MAS.

6.1.1.6. Initiate customer refund using form QA-704-A.

6.1.1.7. Print and sign form QA-705-B. Forward the form to the Product Complaint Coordinator.

6.1.2. Product Complaint Coordinator

6.1.2.1. Verify form QA-705-B for completeness.

6.1.2.2. Verify the accuracy of the information in MAS.

6.1.2.3. Assign a Complaint Number and record information on the Complaint Log form QA-705-D. Also record the Complaint Number on form QA-704-B.

6.1.2.4. Determine the category of AE. If AE is a Serious Adverse Event or a potential Serious Adverse Event, immediately contact AE Management.

6.1.2.5. Update customer file in MAS, if necessary.

6.1.2.6. Complete the Product Complaint Coordinator section of the AER Form and forward to AE Management.

6.1.3. Adverse Event Management

6.1.3.1. Review the information on form QA-705-B. AE Management will determine a severity grade for each AE based upon the details of the individual complaint. The National Cancer Institute-Common Terminology Criteria for Adverse Events (version 4) will be used to provide guidance for severity grading. Any AE that is graded as a 3 or greater will be investigated.

6.1.3.1.1. **All Serious Adverse Events must be investigated.** If found to be a valid SAER, it must be reported to the FDA within 15 days of receiving the information per QA-706.

6.1.3.2. Any returned product will be received by H&D per QA-704 and transferred to Quality Management.

6.1.3.3. Determine whether further information is required from the reporter and contact the reporter to gather additional information as required.

6.1.3.4. Determine necessary investigational activities. This may include a historical complaint review, internal batch record review, contract facility investigation, returned and/or reserve product testing, evaluation of additional impacted lots, and inventory assessment.

6.1.3.5. Document complaint investigation summary on form QA-705-A.

6.1.3.5.1. A Microsoft Word template of this form may be used. The form is not considered official until it is printed out, signed, and dated by AE Management.

6.1.3.6. All completed complaint investigation summary forms QA-705-A will be reviewed by Quality Management.

6.1.3.7. If the reporter requests a response, AE Management will draft and send an investigational summary letter.

6.1.3.8. AE form QA-705-B, complaint investigation form QA-705-A, and any additional documentation associated with each adverse event will be retained by AE Management for 6 years after the report is received.

6.1.3.9. Any corrective and preventive actions (CAPA) generated as a result of the complaint investigation will be assigned to individual site personnel where appropriate to facilitate and/or complete the required actions.

6.1.3.10. Determine on a monthly basis the number of adverse events reported for all product lot numbers and initiate an investigation for any lot where the percentage of adverse events exceeds 0.5% of the amount of that lot in distribution.

6.1.3.11. Perform trend analysis quarterly and evaluate for trends/systematic quality issues. A memo is maintained capturing this evaluation.

6.2. Manufacturing Defects

6.2.1. Receipt of Complaint

6.2.1.1. Upon receipt of a possible manufacturing defect, the complaint recipient captures the initial information from the reporter by completing the Complaint Recipient section of form QA-705-C.

6.2.1.1.1. A Microsoft Word template of this form will be used to type information into the Complaint Recipient section. The form is not considered official until it is printed out, signed, and dated by the complaint recipient.

6.2.1.2. Request that the reporter return the product for investigation. Inform the reporter that they will be receiving a pre-paid envelope to return their product.

6.2.1.3. Enter lot number, complaint description, and any other applicable information in a customer memo in MAS.

6.2.1.4. Initiate customer refund using form QA-704-A.

6.2.1.5. Print and sign form QA-705-C. Forward the form to the Product Complaint Coordinator.

6.2.2. Product Complaint Coordinator

6.2.2.1. Verify form QA-705-C for completeness.

6.2.2.2. Verify the accuracy of the information recorded in MAS.

6.2.2.3. Assign a Complaint Number and record information on the Complaint Log form QA-705-D. Also record the Complaint Number on form QA-704-B.

6.2.2.4. Update customer file in MAS, if necessary.

6.2.2.5. Complete the Product Complaint Coordinator section of the MD Form and forward to the Manufacturing Defects Coordinator.

6.2.3. Manufacturing Defects Coordinator

6.2.3.1. Review the information on form QA-705-C to determine whether the complaint involves a possible failure of a dietary supplement to meet any of its specifications, or any other requirements of 21CFR111, including those specifications and other requirements that, if not met, may result in a risk of illness or injury.

6.2.3.2. Any returned product will be received by H&D per QA-704 and transferred to Quality Management.

6.2.3.3. Investigate any complaint that involves a possible failure of a dietary supplement to meet any of its specifications, or any other requirements of 21CFR111, including those specifications and other requirements that, if not met, may result in a risk of illness or injury.

6.2.3.4. Determine whether further information is required from the reporter and contact the reporter to gather additional information as required.

6.2.3.5. Determine necessary investigational activities. This may include a historical complaint review, internal batch record review, contract facility investigation, returned and/or reserve product testing, evaluation of additional impacted lots, and inventory assessment.

6.2.3.6. Document complaint investigation summary on form QA-705-A.

6.2.3.6.1. A Microsoft Word template of this form may be used. The form is not considered official until it is printed out, signed, and dated by the MD Coordinator.

6.2.3.7. All completed complaint investigation summary forms QA-705-A will be reviewed by Quality Management.

6.2.3.8. If the reporter requests a response, the MD coordinator will draft and send an investigational summary letter.

6.2.3.9. MD form QA-705-C, complaint investigation form QA-705-A, and any additional documentation associated with each manufacturing defect will be retained by the MD Coordinator. Records will be retained for 2 years beyond the date of distribution of the last batch of dietary supplements associated with the records.

6.2.3.10. Any corrective and preventive actions (CAPA) generated as a result of the complaint investigation will be assigned to individual site personnel where appropriate to facilitate and/or complete the required actions.

6.2.3.11. Perform trend analysis quarterly and evaluate for trends/systematic quality issues. A memo is maintained capturing this evaluation.

7. CHANGE HISTORY

Prior Version Number	Summary of Change
None	New
v1	Corrected format, added new instructions to fit GMP Guidelines, Updated forms AB
v2	Changed SOP title to Complaint Handling and Investigation. Added clarification to complaint handling procedure. Added form QA-705-B, QA-705-C, and QA-705-D. Changed form QA-705-A title to Complaint Investigation Form. Retired form QA-705-L and QA-705-AL.
v3	Added procedure for adverse event grading to the SOP and form QA-705-B. Added adverse event 0.5% threshold for investigation of a lot. Added customer number to form QA-705-D.
v4	Changed Accounting duties to Holding & Distribution. Clarified manufacturing defects complaints that need to be investigated.

8. DOCUMENT APPROVALS

		Signature	Date
Revised by:	Mark Roeder Technical and Quality Manager	(b) (6)	[illegible]
Approved by:	Mark Y. Underwood President		10/2/13
Approved by:	Mark Roeder Technical and Quality Manager		[illegible]

QUINCY BIOSCIENCE	Standard Operating Procedures Form ID # **QA-705-A v5**
Replaces Version: v4	Effective Date: **October 9, 2013**
Title: **Complaint Investigation Form**	

Initiated By: _____	**Complaint Number: CN-**_____	**AER** ☐ **MD** ☐
Lot #: _____	**Customer number:** _____	**RMA Number:** _____

CORRECTIVE ACTION ☐**Yes** ☐**No**	**CAPA Number:** _____

Approvals:

Manufacturing Defects Coordinator:	Signature	Date:
AE Management:	Signature	Date:
Quality Management:	Signature	Date:

QUINCY BIOSCIENCE	Standard Operating Procedures Form ID # **QA-705-B v5**
Replaces Version: v4	Effective Date: **October 9, 2013**

Adverse Event Report (AER) Form

COMPLAINT RECIPIENT

Date: 1 October 2013 Inbound Call ☐ Outbound Call ☐ Inbound Shipment ☐

Staff Member Taking Call/Shipment: _____

Customer Name: _____ Customer Phone: _____

If caller is not customer: Caller Name: _____ Caller Phone: _____Relationship to Customer:_____

Sex: Choose One Product: **Select from list** **If Other** _____ Where did you purchase your product? _____

Customer Number: _____ **Lot Number:** _____

What exactly are you experiencing? _____

How many capsules are/were you taking? _____ Have you stopped taking the product? **Select from list**

When did this start? _____ How are you feeling now? _____

Did you seek medical attention for this issue? **Select from list**

Do you take other supplements or prescription medications? **Select from list** If yes, please list: _____

Do you have any allergies? **Select from list** If yes, what are they? _____

Do you have any ongoing medical conditions/concerns? **Select from list** If yes, please describe? _____

Is there anything else in your normal routine that you've changed since starting on our product? _____

Other comments: _____

AER Severity/Action: Select from list **Return of product requested? Select from list**

Signature: _____ Date: _____

PRODUCT COMPLAINT COORDINATOR

Adverse Event ☐ **Serious Adverse Event** ☐

CN-_____

MAS Verified: ☐ Comments: _____

_____

Signature: _____ Date: _____

ADVERSE EVENT MANAGEMENT Adverse Event Term: _____

Adverse Event Severity Grade:

1(Mild) ☐ **2(Moderate)** ☐ **3(Severe)** ☐ **4(Life Threatening)** ☐ **5(Death)** ☐

Will an investigation be initiated? Yes ☐ No ☐ **If yes, complete form QA-705-A.**

Comments: _____

_____

_____

Adverse Event ☐ **Serious Adverse Event** ☐

Signature: _____ Date: _____

QUINCY BIOSCIENCE	Standard Operating Procedures Form ID # **QA-705-C v5**
Replaces Version: v4	Effective Date: **October 9, 2013**

Manufacturing Defect (MD) Form

Date: 1 October 2013 Inbound Call ☐ Outbound Call ☐ Inbound Shipment ☐

Staff Member Taking Call/Shipment: _____

Customer Name: _____ Customer Phone: _____

If caller is not customer: Caller Name: _____ Caller Phone: _____ Relationship to Customer: _____

Sex: Choose One Product: **Select from list** If Other _____

Where did you purchase your product? _____

Customer Number: _____ **Lot Number:** _____

Type of Manufacturing Defect: **Select from list** If Other _____ Comments: _____

Description of Defect: _____ **Return of product requested:** **Select from list**

Comments: _____

Signature: _____ Date: _____

PRODUCT COMPLAINT COORDINATOR

CN-_____

MAS Verified: ☐ Lot Number: ☐ Comments: _____

_____

_____

Signature: _____ Date: _____

MANUFACTURING DEFECTS COORDINATOR

Comments: _____

_____

_____

_____

Will an investigation be initiated? Yes ☐ No ☐ **If yes, complete form QA-705-A.**

Signature: _____ Date: _____

QUINCY	Standard Operating Procedures Form ID #: **QA-705-D v5**
Replaces Version: v4	Effective Date: **October 9, 2013**
Title: **Complaint Log**	

Complaint Number	Date Assigned	AER/SAER/ Manufacturing Defect (MD)	Product	Lot Number	Description (Include customer number)	Assigned By

QA Review: _____ Date: _____

000150

QUINCY	Standard Operating Procedures ID # **QA-706 v3**
Replaces Version: v2	Effective Date: **July 26, 2011**
Title: **Serious Adverse Event Reporting (SAER)**	

1. **PURPOSE**
   1.1. The purpose of this document is to provide a general procedure describing how serious adverse events are reported to the FDA.

2. **SCOPE**
   2.1. This document applies to any Quincy Bioscience (QB) employee or additional associates who are involved with reporting serious adverse events.

3. **RESPONSIBILITIES**
   3.1. **Adverse Event Management**: Primary point of contact, along with corporate officers, with responsibility for notifying appropriate regulatory agencies (ie: the FDA) when a serious adverse event is reported to Quincy Bioscience.

4. **DEFINITIONS**
   4.1. **Dietary Supplement Product (Product)**: Any dietary supplement item sold, distributed, or provided to a customer by Quincy Bioscience.
   4.2. **Serious Adverse Event**: An adverse event is any undesirable experience associated with the use of product. The event is serious and should be reported when the event is:
      4.2.1. Death: report if the patient's death is suspected as being a direct outcome of the adverse event.
      4.2.2. Life-Threatening: report if the patient was at substantial risk of dying at the time of the adverse event, or it is suspected that the use or continued use of the product would result in the patient's death.
      4.2.3. Hospitalization (initial or prolonged): report if admission and detainment to the hospital or prolongation of a hospital stay results because of the adverse event.
      4.2.4. Disability: report if the adverse event resulted in a significant, persistent, or permanent change, impairment, damage or disruption in the patient's body function/structure, physical activities or quality of life.
      4.2.5. Congenital Anomaly: report if there are suspicions that prolonged exposure to a product prior to conception or during pregnancy resulted in an adverse outcome in the child.
   4.3. **Complaint**: Any complaint or expression of dissatisfaction with a product. Can be related to either an Adverse Event or a Manufacturing Defect.
   4.4. **Reporter**: Person or institution making an adverse event report (Customer, Doctor, or Hospital personnel, for example).
   4.5. **Attachments**: Copies of customer correspondence, written, or electronic notes regarding verbal communication with customers, reports, records of investigations, or any completed forms.

5. **REFERENCES AND DOCUMENTATION**
   5.1. Complaint Handling and Investigation (QA-705)

6. **PROCEDURE**
   6.1. Upon receiving a report of a Serious Adverse Event as defined in section 4.1, the SAE is investigated per QA-705.

Issued to: West-Field
QA: (b) (6)
Date: 11/20/11

EXACT COPY

6.2. If after the conclusion of investigation, the adverse event is still deemed to be serious due to the use of product, AE Management, along with officers, obtains MedWatch Form FDA 3500A from the FDA website.

6.3. Along with officers, AE Management completes MedWatch Form FDA 3500A following the instructions on the form.

6.4. The completed MedWatch Form FDA 3500A is submitted to the FDA.

6.4.1. **Serious Adverse Event Reports must be submitted to the FDA within 15 days of receiving the information.**

7. CHANGE HISTORY

Prior Revision Number	Summary of Change
None	New
v1	Corrected format, added new instructions to fit GMP Guidelines, Updated forms AB
v2	Made specific to SAER mandatory reporting and added reference to complaint investigation SOP. Retired form QA-706-A and added reference to FDA website for Form FDA 3500A.

8. DOCUMENT APPROVALS

		Signature	Date
Revised by:	Mark Roeder Technical and Quality Manager	(b) (6) [redacted]	18Jul2011
Approved by:	Mark Y. Underwood President	[redacted]	2/18/4
Approved by:	Mark Roeder Technical and Quality Manager	[redacted]	18Jul2011

Exhibit 2

Adverse Event	#	%	AERs / Bottles
Headaches	428	18.76%	One AER for Every 4,588
Dizziness	172	7.54%	One AER for Every 11,416
Nausea	155	6.80%	One AER for Every 12,668
Hypertension	84	3.68%	One AER for Every 23,375
Diarrhea	74	3.24%	One AER for Every 26,533
Memory Impairment	74	3.24%	One AER for Every 26,533
Insomnia	63	2.76%	One AER for Every 31,166
Anxiety	54	2.37%	One AER for Every 36,361
Stomach Pain	51	2.24%	One AER for Every 38,499
Confusion	46	2.02%	One AER for Every 42,684
Urticaria	46	2.02%	One AER for Every 42,684
Allergic Reaction	41	1.80%	One AER for Every 47,889
Rash, Other	40	1.75%	One AER for Every 49,087
Depression	38	1.67%	One AER for Every 51,670
Psych Disorders, Nightmares	34	1.49%	One AER for Every 57,749
Constipation	31	1.36%	One AER for Every 63,338
Pruritus	27	1.18%	One AER for Every 72,721
Somnolence	26	1.14%	One AER for Every 75,518
Lethargy	24	1.05%	One AER for Every 81,811
Pain in Extremity, Leg	20	0.88%	One AER for Every 98,173
Agitation	16	0.70%	One AER for Every 122,717
Fatigue	16	0.70%	One AER for Every 122,717
Paresthesia	16	0.70%	One AER for Every 122,717
Edema, Limb	15	0.66%	One AER for Every 130,898
Epistaxis	15	0.66%	One AER for Every 130,898
Myalgia	15	0.66%	One AER for Every 130,898
Unknown	15	0.66%	One AER for Every 130,898
Blurred Vision	14	0.61%	One AER for Every 140,248
Rash, Maculo-Papular	14	0.61%	One AER for Every 140,248
Tinnitus	14	0.61%	One AER for Every 140,248
Vertigo	13	0.57%	One AER for Every 151,036
Alopecia	12	0.53%	One AER for Every 163,622
Hallucinations	12	0.53%	One AER for Every 163,622
Malaise	12	0.53%	One AER for Every 163,622
Palpitations	12	0.53%	One AER for Every 163,622
Seizure	12	0.53%	One AER for Every 163,622
Cheilitis	11	0.48%	One AER for Every 178,497
Edema	11	0.48%	One AER for Every 178,497
Hot Flashes	11	0.48%	One AER for Every 178,497
Non-Cardiac Chest Pain	11	0.48%	One AER for Every 178,497
Edema, trunk	10	0.44%	One AER for Every 196,347
Dry Mouth	9	0.39%	One AER for Every 218,163
Neck Pain	9	0.39%	One AER for Every 218,163
Tremors	9	0.39%	One AER for Every 218,163
Arthralgia	8	0.35%	One AER for Every 245,433
Back Pain	8	0.35%	One AER for Every 245,433

Chest Pain, Cardiac	8	0.35%	One AER for Every 245,433
Myalgia, Leg	8	0.35%	One AER for Every 245,433
Skin, Other	8	0.35%	One AER for Every 245,433
Brain fog	7	0.31%	One AER for Every 280,495
Gastroesophageal Reflux	7	0.31%	One AER for Every 280,495
Hyperglycemia	7	0.31%	One AER for Every 280,495
Hypotension	7	0.31%	One AER for Every 280,495
Vomiting	7	0.31%	One AER for Every 280,495
Blue Perspiration	6	0.26%	One AER for Every 327,245
Dry Eye	6	0.26%	One AER for Every 327,245
MS Exacerbation	6	0.26%	One AER for Every 327,245
Oral Dysesthesia	6	0.26%	One AER for Every 327,245
Pain	6	0.26%	One AER for Every 327,245
Rapid Heartbeat	6	0.26%	One AER for Every 327,245
Felt Funny	5	0.22%	One AER for Every 392,693
Flushing	5	0.22%	One AER for Every 392,693
Shortness Of Breath	5	0.22%	One AER for Every 392,693
Spacey	5	0.22%	One AER for Every 392,693
Weight Loss	5	0.22%	One AER for Every 392,693
Atrial Fibrillation	4	0.18%	One AER for Every 490,867
Chest Wall Pain	4	0.18%	One AER for Every 490,867
Cognitive Disturbance	4	0.18%	One AER for Every 490,867
Concentration Impairment	4	0.18%	One AER for Every 490,867
Dysesthesia	4	0.18%	One AER for Every 490,867
Dyspenia	4	0.18%	One AER for Every 490,867
Increased Appetite	4	0.18%	One AER for Every 490,867
Irritability	4	0.18%	One AER for Every 490,867
Oral Blisters	4	0.18%	One AER for Every 490,867
Pre-syncope	4	0.18%	One AER for Every 490,867
Syncope	4	0.18%	One AER for Every 490,867
Tingling	4	0.18%	One AER for Every 490,867
Arrhythmia	3	0.13%	One AER for Every 654,489
Blood Disorders Other. Thin Blood	3	0.13%	One AER for Every 654,489
Dry Skin	3	0.13%	One AER for Every 654,489
Ear Pain	3	0.13%	One AER for Every 654,489
Esophageal Pain	3	0.13%	One AER for Every 654,489
Euphoria	3	0.13%	One AER for Every 654,489
Eye Disorder, Visual Disturbance	3	0.13%	One AER for Every 654,489
Eye Pain	3	0.13%	One AER for Every 654,489
flatulence	3	0.13%	One AER for Every 654,489
Hematuria	3	0.13%	One AER for Every 654,489
Herpes flare	3	0.13%	One AER for Every 654,489
Hypersomnia	3	0.13%	One AER for Every 654,489
Kidney Stones	3	0.13%	One AER for Every 654,489
Mania	3	0.13%	One AER for Every 654,489
Muscle Cramping	3	0.13%	One AER for Every 654,489
Nasal Congestion	3	0.13%	One AER for Every 654,489

Restlessness	3	0.13%	One AER for Every 654,489
Sinusitis	3	0.13%	One AER for Every 654,489
Sore Throat	3	0.13%	One AER for Every 654,489
Stroke	3	0.13%	One AER for Every 654,489
Aches	2	0.09%	One AER for Every 981,734
Amnesia	2	0.09%	One AER for Every 981,734
Anorexia	2	0.09%	One AER for Every 981,734
Balance	2	0.09%	One AER for Every 981,734
Bruising	2	0.09%	One AER for Every 981,734
Creatinine Increased	2	0.09%	One AER for Every 981,734
Dyspepsia	2	0.09%	One AER for Every 981,734
Dysphagia	2	0.09%	One AER for Every 981,734
Facial Blisters	2	0.09%	One AER for Every 981,734
Facial Numbness	2	0.09%	One AER for Every 981,734
Gait Disturbance	2	0.09%	One AER for Every 981,734
Generalized Muscle Weakness	2	0.09%	One AER for Every 981,734
Hyperhydrosis	2	0.09%	One AER for Every 981,734
Hyperuricemia	2	0.09%	One AER for Every 981,734
Irregular Heartbeat	2	0.09%	One AER for Every 981,734
Libido Decreased	2	0.09%	One AER for Every 981,734
Mucositis, Oral	2	0.09%	One AER for Every 981,734
Neuralgia	2	0.09%	One AER for Every 981,734
Parkinson's Disease Excacebation	2	0.09%	One AER for Every 981,734
Personality Change	2	0.09%	One AER for Every 981,734
Renal and Urinary, Other	2	0.09%	One AER for Every 981,734
Renal Colic	2	0.09%	One AER for Every 981,734
Swollen eyes	2	0.09%	One AER for Every 981,734
Tightness In Throat	2	0.09%	One AER for Every 981,734
Transient Ischemic Attack	2	0.09%	One AER for Every 981,734
Urinary Tract Infection	2	0.09%	One AER for Every 981,734
Urinary Tract Pain	2	0.09%	One AER for Every 981,734
Urine Frequency	2	0.09%	One AER for Every 981,734
Vaginal hemorrhage	2	0.09%	One AER for Every 981,734
Vitreous Hemorrhage	2	0.09%	One AER for Every 981,734
Warmth	2	0.09%	One AER for Every 981,734
Weight Gain	2	0.09%	One AER for Every 981,734
Yeast Infection	2	0.09%	One AER for Every 981,734
"Adverse Effects"	1	0.04%	One AER for Every 1,963,467
"Felt Bad"	1	0.04%	One AER for Every 1,963,467
"Felt Drunk/Med. Interaction"	1	0.04%	One AER for Every 1,963,467
Abdominal Pain	1	0.04%	One AER for Every 1,963,467
Achy Joints	1	0.04%	One AER for Every 1,963,467
Achy Muscles	1	0.04%	One AER for Every 1,963,467
Affected Blood Sugar Levels	1	0.04%	One AER for Every 1,963,467
Aging	1	0.04%	One AER for Every 1,963,467
Arm Tingling	1	0.04%	One AER for Every 1,963,467
Benign Papilloma	1	0.04%	One AER for Every 1,963,467

Bitter Fluid Back In Throat	1	0.04%	One AER for Every 1,963,467
Bladder Infection	1	0.04%	One AER for Every 1,963,467
Bleeding gums	1	0.04%	One AER for Every 1,963,467
Blepharospasm	1	0.04%	One AER for Every 1,963,467
Bloating	1	0.04%	One AER for Every 1,963,467
Blood Clots-Placebo	1	0.04%	One AER for Every 1,963,467
Blood Red Spot On Forehead	1	0.04%	One AER for Every 1,963,467
Blood Thickening	1	0.04%	One AER for Every 1,963,467
Bloody Mucus	1	0.04%	One AER for Every 1,963,467
Blue Hair	1	0.04%	One AER for Every 1,963,467
Blue Mucus	1	0.04%	One AER for Every 1,963,467
Body Odor	1	0.04%	One AER for Every 1,963,467
Brain Neoplasm NOS	1	0.04%	One AER for Every 1,963,467
Bruxism	1	0.04%	One AER for Every 1,963,467
Bubbles In Brain	1	0.04%	One AER for Every 1,963,467
Bumps On Leg	1	0.04%	One AER for Every 1,963,467
Burning Sensation In Breast	1	0.04%	One AER for Every 1,963,467
Burning/Swelling In Mouth	1	0.04%	One AER for Every 1,963,467
Calcium Thru The Skin..!	1	0.04%	One AER for Every 1,963,467
Cataract	1	0.04%	One AER for Every 1,963,467
Change In Stool Color	1	0.04%	One AER for Every 1,963,467
Chills	1	0.04%	One AER for Every 1,963,467
Claimed She Felt Funny	1	0.04%	One AER for Every 1,963,467
Cold Legs	1	0.04%	One AER for Every 1,963,467
Colitis	1	0.04%	One AER for Every 1,963,467
Colored Perspiration	1	0.04%	One AER for Every 1,963,467
Congenital Disorder	1	0.04%	One AER for Every 1,963,467
Couldn't Speak	1	0.04%	One AER for Every 1,963,467
Created Growths On Face	1	0.04%	One AER for Every 1,963,467
CV	1	0.04%	One AER for Every 1,963,467
Cyanosis, Lips	1	0.04%	One AER for Every 1,963,467
Oifficulty Walking	1	0.04%	One AER for Every 1,963,467
Oiscolored feces	1	0.04%	One AER for Every 1,963,467
Driving Problems	1	0.04%	One AER for Every 1,963,467
Drum Sensation In His Teeth	1	0.04%	One AER for Every 1,963,467
Dysarthia	1	0.04%	One AER for Every 1,963,467
Dysgeusia	1	0.04%	One AER for Every 1,963,467
Eczema	1	0.04%	One AER for Every 1,963,467
Erectile Dysfunction	1	0.04%	One AER for Every 1,963,467
Erythema multiforme	1	0.04%	One AER for Every 1,963,467
Extremity Tingling	1	0.04%	One AER for Every 1,963,467
Eyelid Function Disorder	1	0.04%	One AER for Every 1,963,467
Feeling Abnormal	1	0.04%	One AER for Every 1,963,467
Felt Weak In Her Legs	1	0.04%	One AER for Every 1,963,467
Felt Worse	1	0.04%	One AER for Every 1,963,467
Flank Pain	1	0.04%	One AER for Every 1,963,467
Flashing lights	1	0.04%	One AER for Every 1,963,467

Floaters	1	0.04%	One AER for Every 1,963,467
Flu-like Symptoms	1	0.04%	One AER for Every 1,963,467
Getting A Cold	1	0.04%	One AER for Every 1,963,467
Glioblastoma Multiforme	1	0.04%	One AER for Every 1,963,467
Hair Color Change*	1	0.04%	One AER for Every 1,963,467
Hand Twitching	1	0.04%	One AER for Every 1,963,467
Heaviness In The Head	1	0.04%	One AER for Every 1,963,467
Heavy Eyelids	1	0.04%	One AER for Every 1,963,467
Hernia got worse	1	0.04%	One AER for Every 1,963,467
Hyperthyroidism	1	0.04%	One AER for Every 1,963,467
Hypoglossal Nerve Disorder	1	0.04%	One AER for Every 1,963,467
Hypoglycemia	1	0.04%	One AER for Every 1,963,467
Hyponatremia	1	0.04%	One AER for Every 1,963,467
Hypothyroidism	1	0.04%	One AER for Every 1,963,467
increased energy	1	0.04%	One AER for Every 1,963,467
Increased Pain	1	0.04%	One AER for Every 1,963,467
Infections, Other	1	0.04%	One AER for Every 1,963,467
Intracranial Hemorrhage	1	0.04%	One AER for Every 1,963,467
Investigations, INR Decreased	1	0.04%	One AER for Every 1,963,467
Involuntary Movements	1	0.04%	One AER for Every 1,963,467
Ischemia, Cerebrovascular	1	0.04%	One AER for Every 1,963,467
ITP Levels Went Down	1	0.04%	One AER for Every 1,963,467
Joint Pain	1	0.04%	One AER for Every 1,963,467
Laryngeal Inflammation	1	0.04%	One AER for Every 1,963,467
leg muscle stiffness	1	0.04%	One AER for Every 1,963,467
Lower Leg Foot Cramps	1	0.04%	One AER for Every 1,963,467
Lupus Symptoms	1	0.04%	One AER for Every 1,963,467
Macular Degeneration	1	0.04%	One AER for Every 1,963,467
Made Her "Off Balanced" Feeling	1	0.04%	One AER for Every 1,963,467
Made Her Hungry	1	0.04%	One AER for Every 1,963,467
Made His Ears Bleed	1	0.04%	One AER for Every 1,963,467
Made Liver Enzymes Go Up	1	0.04%	One AER for Every 1,963,467
Magnesium	1	0.04%	One AER for Every 1,963,467
Mammary Gland Sensitivity	1	0.04%	One AER for Every 1,963,467
Menopause	1	0.04%	One AER for Every 1,963,467
Metabolism	1	0.04%	One AER for Every 1,963,467
Mucus In Eyes	1	0.04%	One AER for Every 1,963,467
Muscle Test	1	0.04%	One AER for Every 1,963,467
muscle twitches	1	0.04%	One AER for Every 1,963,467
Muscloskelatal Other	1	0.04%	One AER for Every 1,963,467
Myocardial Infarction	1	0.04%	One AER for Every 1,963,467
Neoplasms, Other	1	0.04%	One AER for Every 1,963,467
Nervous system disorders - Other, specify	1	0.04%	One AER for Every 1,963,467
Nose Tickling	1	0.04%	One AER for Every 1,963,467
Numb Feet And Legs	1	0.04%	One AER for Every 1,963,467
Oral Mucositis	1	0.04%	One AER for Every 1,963,467
Osteoporosis	1	0.04%	One AER for Every 1,963,467

Paralyzed	1	0.04%	One AER for Every 1,963,467
Psoriasis	1	0.04%	One AER for Every 1,963,467
Psychosis	1	0.04%	One AER for Every 1,963,467
Quivering	1	0.04%	One AER for Every 1,963,467
Rash Acneform	1	0.04%	One AER for Every 1,963,467
Rectal Hemorrhage	1	0.04%	One AER for Every 1,963,467
Reported Feeling "Owly."	1	0.04%	One AER for Every 1,963,467
Right Eye Bleeding	1	0.04%	One AER for Every 1,963,467
Seeing spots	1	0.04%	One AER for Every 1,963,467
Sharp Pain	1	0.04%	One AER for Every 1,963,467
Shingles	1	0.04%	One AER for Every 1,963,467
Shopping (?)	1	0.04%	One AER for Every 1,963,467
Sick	1	0.04%	One AER for Every 1,963,467
Sight And Hearing	1	0.04%	One AER for Every 1,963,467
Skin Disorder, Other, Eczema	1	0.04%	One AER for Every 1,963,467
Skin infection	1	0.04%	One AER for Every 1,963,467
Sore	1	0.04%	One AER for Every 1,963,467
Stepping On Glass	1	0.04%	One AER for Every 1,963,467
Suicidal Thoughts	1	0.04%	One AER for Every 1,963,467
Swelling Of Joints	1	0.04%	One AER for Every 1,963,467
Swollen Tongue	1	0.04%	One AER for Every 1,963,467
Teary Eyes	1	0.04%	One AER for Every 1,963,467
Told Not To Take	1	0.04%	One AER for Every 1,963,467
Too Much Energy	1	0.04%	One AER for Every 1,963,467
Tooth Pain	1	0.04%	One AER for Every 1,963,467
Elevated Triglycerides	1	0.04%	One AER for Every 1,963,467
Turned Everything Yellow/Orang	1	0.04%	One AER for Every 1,963,467
Twitching In Eye	1	0.04%	One AER for Every 1,963,467
Urinary Retention	1	0.04%	One AER for Every 1,963,467
Urinary Urgency	1	0.04%	One AER for Every 1,963,467
Uterine Pain	1	0.04%	One AER for Every 1,963,467
Various Adverse Effects.	1	0.04%	One AER for Every 1,963,467
Vestibular Disorder	1	0.04%	One AER for Every 1,963,467
Watery Eyes	1	0.04%	One AER for Every 1,963,467
Weakness In Left Hand	1	0.04%	One AER for Every 1,963,467
Sum	2281		One AER for Every 861

Exhibit 3

AMIN TALATI

ATTORNEYS AT LAW

Rakesh M. Amin, LL.M., R.Ph.
Ashish R. Talati*
Ryan M. Kaiser
Paul L. Brown
Jonathan J. Krit
Janine A. (Jen) Moderson

*Admitted in New York

Abhishek K. Gurnani
Brent A. Batzer
Saira J. Alikhan
Tamara A. Brey
Ashley L. Nelson

George M. Carrera, Jr.
Registered Patent Agent

November 20, 2013

Food and Drug Administration
Division of Freedom of Information
Office of the Executive Secretariat, OC
12420 Parklawn Drive, ELEM-1029
Rockville, MD 20857

Re: Freedom of Information Act (FOIA) Request

Dear Division of Freedom of Information:

Pursuant to Section 20.40 of Title 21 of the Code of Federal Regulations, we are hereby requesting the following records from FDA concerning the product Prevagen® (including the dietary ingredient apoaequorin) and its manufacturer\distributor Quincy Bioscience Manufacturing, Inc. (and any of its subsidiary or affiliated companies) ("Quincy"), located at 301 S. Westfield Road, Suite 200, Madison, Wisconsin 53717:

- Any and all correspondence between FDA and Quincy including but not limited to any warning letters, Form 483 observations, and other correspondence from FDA to Quincy regarding Quincy or its Prevagen® product;
- Any and all internal FDA correspondence regarding Quincy or its Prevagen® product including but not limited to any interagency communications or other correspondence between FDA employees or FDA district offices concerning Quincy or its Prevagen® product;
- Any and all correspondence between FDA and members of the public including but not limited to any complaints received, response letters, or any other written communication between FDA and members of the public concerning Quincy or its Prevagen® product.
- Any and all correspondence between FDA and other State or Federal regulatory agencies concerning Quincy or its Prevagen® product.

Thank you for your assistance. Please do not hesitate to contact me via telephone 312.327.3325 or Abhishek@AminTalati.com if you have any questions or concerns regarding this request.

Sincerely,

(b) (6)

Abhishek K. Gurnani

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
Rockville MD 20857

Abhishek Gurnani
Amin Talati
55 West Monroe Street
Suite 3400
Chicago, IL 60603

F13-9323

Dear Mr. Gurnani:

In response to your request of November 20, 2013 for information pertaining to Prevagen (including the dietary ingredient apoacquorin) and its manufacturer/distributor Quincy Bioscience Manufacturing Inc., Madison, Wisconsin 53717.

XX Enclosed are the records you requested.

____ We have searched our files and find no responsive information.

____ Your request is also being referred to one of our component offices.

____ Certain material has been deleted from the records furnished to you because a preliminary review of the records indicated that the deleted information is not required to be publicly disclosed and that disclosure is not appropriate. FDA has taken this approach to facilitate the process of responding to you. If you dispute FDA's preliminary determination with respect to these records and would like FDA to reconsider any particular deletion, please let us know in writing at the following address: Food and Drug Administration, Division of Freedom of Information, 12420 Parklawn Drive, Room 1050 Rockville, MD 20850 within 30 days from the date of this letter. If we do not receive a response in that time period, we will consider the matter closed with respect to these records. If you do request further consideration and if the agency then formally denies your request for any or all of the previously-withheld information, you would have the right to appeal that decision. Any letter of denial will explain how to make this appeal.

The following charges for this request to date may be included in a monthly invoice:

Reproduction$ 1.30 Search$ 138.00 Review$ 0 Other$ 0 Total:$ 139.30

THE ABOVE TOTAL MAY NOT REFLECT THE FINAL CHARGES FOR THIS REQUEST. PLEASE DO NOT SEND PAYMENT UNLESS YOU RECEIVE AN INVOICE.

(b) (6)

Sincerely yours,

(b) (6)

Government Information Specialist
Executive Secretariat Staff
Office of Foods and Veterinary Medicine/
Center for Food Safety and Applied Nutrition

Enclosure

CAERS Disclaimer

FDA's Center for Food Safety and Applied Nutrition's (CFSAN's) Adverse Event Reporting System (CAERS) is a post-market surveillance system that collects reports about adverse events and product complaints that are allegedly related to CFSAN-regulated products. These products include conventional foods (and beverages), dietary supplements, infant formulas and cosmetics.

The adverse event reports about a product and the total number of adverse event reports for that product in CAERS only reflect information **AS REPORTED** and do not represent any conclusion by FDA about whether the product actually caused the adverse events.

The reports submitted to FDA vary in the quality and reliability of the information provided. Some reports to FDA do not necessarily include all relevant data, such as whether an individual also suffered from other medical conditions or used other products or medications at the same time. Reports may not include accurate or complete contact information for FDA to seek further information about the event, or complainants may choose not to participate in a follow-up investigation. When important information is missing from a report, it is difficult for FDA to fully evaluate whether the product caused the adverse event or simply coincided with it. There also may be duplicate reports in CAERS for the same adverse event because multiple people (such as an injured consumer and a health care provider who treated him or her) may have submitted reports

Because CAERS is constantly updated with new information, the number of reports for a given product and the content of individual reports may change over time.

FOI 13-9323
CAERS Reports Allegedly Related to Prevagen
Search Terms: Prevagen

The Center for Food Safety Adverse Event Reporting System (CAERS) is a post-market surveillance system that collects reports about events or problems that are allegedly related to CFSAN regulated products. In some reports, information in the reports cannot be verified for accuracy of the symptom(s), product(s), or ingredient(s). Furthermore, in many reports, individuals may have used other products, and many products contain multiple ingredients which further complicates the evaluation of adverse event reports.

There is no certainty that a reported adverse event can be attributed to a particular product or ingredient. The number of adverse event reports in CAERS received by FDA and the adverse event report itself about a particular product only reflects information **AS REPORTED** and does not represent any conclusion by FDA regarding a causal relationship or association with the product or ingredient. Due to the continuous inclusion of new or updated information into the CAERS system, reports released from CAERS containing adverse event data may change over time.

Each report received by CAERS regarding an individual that experiences an adverse event is assigned a unique report number (Report #).

^ Additional dates indicate receipt of additional materials on report.

Report #	Received Date	Brand/Product Name	Symptoms	Outcomes
129147	8/17/10	PREVAGEN (APOAEQUORIN) 10 MG	DIZZINESS, ALTERED VISUAL DEPTH PERCEPTION	HOSPITALIZATION
141869^	6/30/11	PREVAGEN (APOAEQUORIN) 10MG	CONVULSION	HOSPITALIZATION
	9/21/11	PREVAGEN (APOAEQUORIN) 10MG	CONVULSION	HOSPITALIZATION
146177	11/29/11	PREVAGEN 10 MG	CONVULSION	HOSPITALIZATION
147289^	12/28/11	PREVAGEN	BRAIN NEOPLASM, CEREBROVASCULAR ACCIDENT, RENAL DISORDER, GOUT, HYPERTENSION, DIABETES MELLITUS, OBESITY, RENAL IMPAIRMENT, THROMBOSIS, GAIT DISTURBANCE, SPEECH DISORDER	HOSPITALIZATION
	2/1/12	PREVAGEN	BRAIN NEOPLASM, CEREBROVASCULAR ACCIDENT, RENAL DISORDER, GOUT, HYPERTENSION, DIABETES MELLITUS, OBESITY, RENAL IMPAIRMENT, THROMBOSIS, GAIT DISTURBANCE, SPEECH DISORDER	HOSPITALIZATION
147887	1/19/12	PREVAGEN ES 20 MG	MYOCARDIAL INFARCTION	LIFE THREATENING, HOSPITALIZATION
147888	1/19/12	PREVAGEN PROFESSIONAL 20 MG	BRADYCARDIA, ARRHYTHMIA, TACHYCARDIA, DIZZINESS	HOSPITALIZATION
147902	1/19/12	PREVAGEN 10 MG	CONVULSION	OTHER SERIOUS (IMPORTANT MEDICAL EVENTS), HOSPITALIZATION
147904	1/19/12	PREVAGEN PROFESSIONAL 40 MG	CEREBROVASCULAR ACCIDENT, GAIT DISTURBANCE, DYSPNOEA, HEADACHE	HOSPITALIZATION

000165

Report #	Received Date	Brand/Product Name	Symptoms	Outcomes
147905	1/19/12	PREVAGEN PROFESSIONAL 10 MG	NEUROLOGICAL SYMPTOM	OTHER SERIOUS (IMPORTANT MEDICAL EVENTS)
147906	1/19/12	PREVAGEN 10 MG	CEREBROVASCULAR ACCIDENT	OTHER SERIOUS (IMPORTANT MEDICAL EVENTS)
147907	1/19/12	PREVAGEN 10 MG	CONVULSION	OTHER SERIOUS (IMPORTANT MEDICAL EVENTS)
147908	1/19/12	PREVAGEN 10 MG	CONVULSION	OTHER SERIOUS (IMPORTANT MEDICAL EVENTS)
148320	1/31/12	PREVAGEN ES	GLIOBLASTOMA MULTIFORME	OTHER SERIOUS (IMPORTANT MEDICAL EVENTS), HOSPITALIZATION
148685	2/8/12	PREVAGEN PROFESSIONAL 40 MG	HYPOTENSION, DEPRESSED LEVEL OF CONSCIOUSNESS, DEPRESSED LEVEL OF CONSCIOUSNESS	HOSPITALIZATION
149110	2/21/12	PREVAGEN	RENAL DISORDER, BLOOD CREATININE INCREASED, DRUG INTERACTION	OTHER SERIOUS (IMPORTANT MEDICAL EVENTS)
149959	3/13/12	PREVAGEN PROFESSIONAL 40 MILIGRAMS	PAPILLOMA, INTRADUCTAL PAPILLOMA OF BREAST, BLOODY DISCHARGE	HOSPITALIZATION
152915	5/31/12	QUINCY BIOSCIENCE PREVAGEN 10 MG	HYPERTENSION, BLOOD PRESSURE INCREASED	OTHER SERIOUS (IMPORTANT MEDICAL EVENTS)
154742	7/25/12	PREVAGEN EXTRA STRENGTH 20 MG PREVAGEN PROFESSIONAL 40 MG	MULTIPLE SCLEROSIS RELAPSE	HOSPITALIZATION
156196	9/6/12	PREVAGEN PROFESSIONAL	ANEURYSM, HAEMORRHAGE INTRACRANIAL	DEATH
162504	2/20/13	PREVAGEN, 10MG, QUINCY BIOSCIENCE	CEREBROVASCULAR ACCIDENT	HOSPITALIZATION, DISABILITY
163742	3/22/13	PREVAGEN 10 MG	OSTEOPOROSIS	OTHER SERIOUS (IMPORTANT MEDICAL EVENTS)
165501	5/13/13	PREVAGEN 10MG	ABDOMINAL PAIN, PAIN	OTHER SERIOUS (IMPORTANT MEDICAL EVENTS)
169807	9/5/13	PREVAGEN, 10 MG	LOSS OF CONSCIOUSNESS, FEELING ABNORMAL, SPEECH DISORDER	HOSPITALIZATION

000166

Report #	Received Date	Brand/Product Name	Symptoms	Outcomes
170983	10/2/13	PREVAGEN EXTRA STRENGTH, 200MG, QUINCY BIOSCIENCE		HOSPITALIZATION, LIFE THREATENING
170984	10/2/13	PREVAGEN, 10 MG, BIOSCIENCE		HOSPITALIZATION
171717	10/30/13	PREVAGEN, 10MG		HOSPITALIZATION
26				

000167

Exhibit 4

REVIEW OF REPORTS OF SERIOUS ADVERSE EFFECTS IN USERS OF PREVAGEN

AUTHOR

Gary M. Williams, MD

New York Medical College
Department of Pathology
Chemical Safety Laboratory
Valhalla, NY 10595

USA

Final Report

24 April 2014

AUTHOR SIGNATURE PAGE

(b) (6)

April 24, 2014

Date (Mo/Day/Year)

Gary M. Williams, MD

Professor of Pathology

Professor of Public Clinical Health

Director, Chemical Safety Laboratory

Department of Pathology

New York Medical College

Valhalla, NY, USA

I. Introduction

Quincy Bioscience markets a dietary supplement under the brand name Prevagen®. The main ingredient in Prevagen® is apoaequorin, a protein comprised of 196 amino acids.

This report deals with adverse effects (AE) of Prevagen reported to the company. Quincy has a rigorous process for capturing and analyzing AEs. This is detailed in the document "Process for Capture and Analysis of Adverse Events Reported to Quincy Bioscience". Initially, the Customer Service Representative determines the severity of the AE. If the AE appears to be a serious AE (SAE) the reporter is transferred to either the Product Complaint Coordinator or Adverse Event Management. Two subsequent levels of review are followed to determine if the AE is a SAE.

Approximately 2,200 AEs have been reported. Most were not serious; for example, headache, dizziness and nausea were the most common. In reviewing the AEs reported, I studied Quincy's investigation and classification process to determine whether AEs are being adequately classified as non-serious or serious. I also reviewed a sampling of AEs which after investigation were classified as non-serious by Quincy. For example, in July of 2013, a customer called Quincy and reported that her systolic blood pressure spiked to 171. The customer reported always having high blood pressure and indicated her "normal" readings are 150/75. The customer also reported that this was an isolated event and not an ongoing issue after consuming Prevagen since September 2012. No medical intervention was reported during the complaint

investigation. Therefore, in my opinion, this AE was properly classified as non-serious by Quincy. In another instance, a customer reported an instance of low blood pressure. The customer had been taking Prevagen for almost two years. The customer stated that her husband "had mixed up some other medications that she takes for diabetes." Based on the information received from the customer after the investigation, Quincy classified this as a non-serious adverse event. Again, in my opinion, this AE was adequately classified as non-serious. From a review of samples of AEs and the AE summary chart, there is no alarming pattern of SAEs which would indicate a safety concern.

Between 2010 and 2013, Quincy recorded 26 instances of SAEs which were reported to the Food and Drug Administration, a mandatory requirement regardless of a link between the product and the SAE reported. Quincy Bioscience requested a detailed review of the 26 SAE reports. Five reports were made in 2010, ten in 2011, four in 2012 and seven in 2013. These are summarized in Table 1 from Quincy. I have reviewed the Complaint Investigation Forms of these reports and my opinions are provided in this report.

II. Adverse Reports

The information in the Complaint Investigation Forms is organized according to year and number assigned in Table 1.

A. 2010

1) A 7 year old male was reported by his father to have experienced seizures different from his normal seizures. History of Prevagen use 1-3 weeks, one 10 mg capsule per day. Child is epileptic and medications were being changed at the time of seizures.

   Event most likely due to pre-existing condition and medication change.

2) An 82 year old female was reported by her daughter to have experienced a seizure. Event occurred after taking one Prevagen 10 mg capsule per day for one week. No previous history of seizures. Individual takes neuroactive medications.

   Cause of event unclear, but could be due to age and medications.

3) An 80 year old female reported an episode of dizziness and falling. Began taking 10 mg capsules of Prevagen 4 – 5 weeks before event. Individual has Parkinson's disease.

   Event likely related to age and pre-existing condition.

4) A 73 year old male was reported by his wife to be experiencing irregular heart rate and dizziness for 1.5 months. Reported using Prevagen 10 mg capsules for 5 months. Condition started after 3-5 months of use. Individual on several medications.

Event likely related to age and pre-existing condition.

5) A 65 year old female reported experiencing a "mini-stroke". Treatment was not sought. History of Prevagen use not available. Individual ordered 3 month and 6 month supplies after event. Relevant medical history not available.

Nature of event unclear, but did not recur with continued use.

B. <u>2011</u>

6) A female of unknown age (old enough to have an adult daughter) reported an increase in frequency of seizures. Individual had product for approximately 6 months and was reported to take one 10 mg capsule per day. Individual has a history of seizures and is under care of a neurologist.

Event likely due to pre-existing condition.

7) A male of unknown age was reported by his daughter to have experienced worsening of chronic inflammatory demyelinating polyneuropathy which the individual had for 10 years. Individual took one 10 mg capsule per day for unspecified duration. Individual

was undergoing radiation therapy for brain tumor at the time of event.

Event likely represents natural progression of condition.

8) A female of approximately 55 years of age was reported by a friend to have experienced a seizure with falling. Individual took one 10 mg capsule every other day. Approximately 30 years previously, the individual had a concussion followed by persistent memory loss and falling almost daily.

Event likely due to pre-existing condition.

9) An 81 year old male was reported by his daughter to have experienced a stroke. Reported Prevagen use of 40 mg capsules, one per day, for about 2 weeks. Individual had risk factors for stroke.

Event likely due to pre-existing condition.

10) A male of unknown age was reported by his wife to have experienced a seizure. Event occurred after taking two 10 mg Prevagen capsules for 2 days. Individual had a stroke 10 years earlier.

Event likely due to pre-existing condition.

11) An 81 year old male was reported by his wife to have a brain tumor. Took 10 mg capsules of Prevagen, but pattern of use not given. Individual has history of strokes.

Event not related to Prevagen use.

12) A 67 year old male was reported by his wife to have experienced a myocardial infarct. Individual took one 20 mg capsule per day for approximately 9 months. Individual had coronary artery disease treated by bypass surgery.

Event not related to Prevagen use.

14) A 52 year old female reported experiencing hypotension. Individual took one 40 mg capsule per day for unspecified duration. Individual has multiple sclerosis and hypotension.

Event likely due to pre-existing condition.

15) A 54 year old female reported a benign mammary gland neoplasm. Individual took one 40 mg capsule per day in a clinical trial.

Event not related to Prevagen use.

19) A 70 year old male had a stroke. Individual participated in Madison Memory trial in 2010. Individual took Prevagen 10 mg capsules for unspecified duration. Individual was informed that he had atrial fibrillation.

Event likely due to pre-existing condition.

C. **2012**

13) A 58 year old male was reported by his wife to have experienced a seizure. Individual took six 20 mg capsules (one per day) while in Memory case study. Individual has history of brain tumor and seizures.

Event due to pre-existing condition.

16) A male of unknown age reported experiencing hypertension. Prevagen 10 mg capsules taken for 1 week. History of pre-existing hypertension.

Event likely due to pre-existing condition.

17) A 43 year old female reported two relapses of multiple sclerosis. Used Prevagen 20 mg capsules for 3 months, 40 mg for 9 months, and 60 mg for 2 months.

History of multiple sclerosis for greater than 8 years.

18) A 90 year old female was reported by her son to have died. History of Prevagen use vague. Individual had history of cardiovascular disease.

Event likely due to pre-existing condition.

D. <u>2013</u>

20) An 80 year old female reported passing out after consuming Prevagen. The history of Prevagen use was not provided. The patient was taking 6 medications.

Event due to other factors.

21) An 80 year old female was reported by her daughter to have been admitted to a hospital for hypercalcemia. Prevagen 20 mg capsules reported to be used for 20 days. "Symptoms" of hypercalcemia preceded Prevagen use.

Event not due to Prevagen.

22) An 85 year old female was reported by her husband to have experienced chest pain. Individual was taking two 10 mg Prevagen capsules daily for 1 month. Individual was found to have coronary artery disease treated by angioplasty.

Event likely related to pre-existing condition.

23) An 85 year old female reported experiencing nausea, vomiting and flatulence. Individual was taking Prevagen 10 mg capsules for about one month prior to event and resumed after event. Individual reported history of colitis.

Event not likely due to Prevagen.

24) An 85 year old male reported experiencing a slight stroke. Individual was taking Prevagen 20 mg capsules. Individual was taking medications for cardiovascular conditions.

Event likely related to pre-existing condition.

25) An 85 year old female was reported by her daughter to have experienced chest pain, irregular heart rate and elevated blood pressure. Individual was taking Prevagen 10 mg capsules. Event occurred 3 weeks after receiving most recent order. History of elevated blood pressure.

Event likely related to pre-existing condition.

26) An 86 year old female was reported by her daughter to have had a stroke. Individual was taking Prevagen 10 mg capsules twice a day intermittently for an unspecified duration. Individual has history of heart palpitations.

Event likely related to pre-existing condition.

III. Conclusions:

Apoaequorin, the main ingredient in Prevagen®, is a protein which is presumably broken down in the gastro-intestinal tract to amino acids. There is nothing in its structure or preclinical testing to suggest any potential toxicities.

The events reported were almost all anecdotal and mainly neurologic or cardiovascular. They show no commonality in presentation or duration of exposure preceeding event.

Some events (e.g. neoplasia) are not attributable to Prevagen because duration of use too short to lead to event.

Most events were associated with pre-exisiting chronic conditions and are attributable to those conditions..

No increase in reports has occurred in spite of accrual of users which makes causality of events by Prevagen unlikely.

Exhibit 5

REVIEW OF REPORTS OF SERIOUS ADVERSE EVENTS IN USERS OF PREVAGEN

Xavier Pi-Sunyer, MD, MPH

Professor of Medicine

Columbia University College of Physicians and Surgeons

New York, NY 10032

May 30, 2014

PRODUCT

Prevagen contains apoaequorin, which is a protein composed of 196 amino acids. The chemical structure is known and contains 20 different amino acids. It is sold as a brain health supplement to improve memory. The protein was originally identified in certain species of jelly fish. The protein is now made via recombinant DNA in E coli by Quincy BioScience. It has been tested in rats for toxicity, with no reported ill effects.

BACKGROUND

Some 2200 adverse events (AEs) have been reported to the company. Some adverse events reported included chest pain, seizures, tremors, fainting, dizziness, nausea, headaches, and allergic reactions such as itching or skin rashes. These reports were categorized as either "serious" or "non-serious" adverse events. At the request of Quincy, I have reviewed 26 instances of serious adverse events (SAEs) and a representative sampling of non-serious adverse events. In my opinion, Quincy's classification of "serious" or "not-serious" bends on the side of calling more events serious than really are. In reviewing the over-all data, it is my opinion that the product Prevagen is safe. I found no pattern of events that led me to think there was a particular safety problem with the product.

SUMMARY OF SERIOUS ADVERSE EVENTS

Quincy reported 26 SAEs to the FDA between 2010 and 2013. I have reviewed these below.

1. Pt#40-83965; MFR# 40-83965 (2010)

7 yr old boy who was an epileptic. The report is that he experienced seizures different from usual. He was on multiple medications including Keppra, Banzel, Lamictal, Depakote. His anti-epileptic regimen was being changed around with differing anti-epileptic medications. He has taken Prevagen for 1-3 weeks, one 10 mg capsule/day.

Impression: AE is most likely due to his long history of difficult epileptic seizures and changes of medications and not to the Prevagen.

This 82 year old woman experienced a seizure. She gave a history of lung cancer and is on Zoloft, Aricept, and also sleeping pills.

Impression: Not likely this was related to Prevagen.

3. Pt# MS-84421; QBSAER-0001 (2010)

80 year old woman who fell and fractured her hip. Question whether dizziness, poor depth perception, or loss of consciousness could have caused this fall. Patient has Parkinson's Disease and is on medications for this and also for hypertension, arthritis, and colitis. On 10 mg Prevagen for a few weeks.

Impression: She has a number of chronic pre-existing -conditions. Not likely that this was caused by Prevagen.

4. Pt#10-77441; MFR# QBSAER-0001 (2010)

73 year old man with arrhythmias and dizziness for 1.5 months; both bradycardia and tachycardia described; given Mixelitine to slow heart rate; also takes Lyrica and Lunesta; On Prevagen 10 mg for 5 months.

Impression: A number of chronic pre-existing conditions. No evidence that taking Prevagen was related to heart condition.

5. Pt#40-1070492; MFR# 4070492 QA-705-A v4 (2010)

65 year old woman reported having a "mini stroke". Contacted afterward and she said she was fine and continuing to take Prevagen. This has been classified by Quincy as an SAE but I doubt it was. When and how much Prevagen she was taking not available in records.

Impression: not an SAE. No evidence that Prevagen caused this problem.

6. Pt#40-103221; MFR 40-103221 (2010)

A woman reporting more frequent seizures. Patient has a history of seizures. No intervention for present complaint. On Prevagen one 10 mg/day.

Impression: She has a pre-existing chronic condition. Unlikely Prevagen is responsible for complaint.

7. Pt#40-98996; MFR40-98996 (2011)

Patient complained of a flare-up of chronic inflammatory demyelinating polyneuropathy. Essentially "paralyzed". Patient also receiving radiation therapy for brain tumor. Doctor believes the symptoms are progression of his disease. Patient on one 10 mg capsule Prevagen.

Impression: Chronic severe pre-existing conditions. Unlikely Prevagen caused the problems.

8. Pt#40-0109704; QBSAER-00002 (2011)

55 yr old woman who had a seizure and fell down. History of motorcycle accident with concussion 30 years ago. 6 month history of almost daily falling. Patient on Elavil, pain killers, and a variety of herbs and supplements. No other history available. Took one 10 mg Prevagen every other day.

Impression: too little information to come to any conclusion, but it seems unrelated to Prevagen.9. Pt#10-109597; MFR# CN-110038 (2011)

81 yr old patient suffered a stroke and hospitalized. Previous history of risk factors including diabetes, hypertension, hyperlipidemia and "small vessel disease of the brain"; headaches, trouble talking, trouble walking; had dementia and diabetes and hypertension; on many medications: janumet, lisinopril, synthroid, methotrexate, plavix, filate, aspirin, potassium, B12, flomax, DHEA, vit D3, cranberry extract; radiation implants in prostate; one 40 mg cap/day of Prevagen.

Impression: no evidence that taking Prevagen is related to his stroke and other conditions.

10. Pt#10-40-0093338; MFR# CN-110155 (2011)

Patient reported having a seizure. Patient had history of a stroke 10 years prior and open heart surgery 5 months prior. Patient on Coumadin and metoprolol. Seizure occurred after he took two 10 mg capsules for two days.

Impression: Not much information, but preexisting stroke history suggests pre-existing cerebral disease. Unlikely that this is related to Prevagen.

11. Pt#40-107059; MFR CN-110194 (2011)

81 year old male with pre-existing gout. Also had hypertension and diabetes with renal dialysis. Also brain tumor and "large blood clot". Medications included Allopurinol.Colchicine, Insulin, and several other medications for hypertension including diuretics. Taking 10 mg Prevagen/day.

Impression: Pre-existing chronic conditions unlikely related to Prevagen.

12. Pt#40-91240; MFR# CN-113216(2011)

67 yr old man with myocardial infarction who was treated with "quadruple bypass procedure". Patient had risk factors for myocarfdial infarction (hyperlipidemia and hypertension). Patient was on Methotrexate, Lisinopril, Sulfazene, and Diclofenic. He was taking one 20 mg capsule of Prevagen/day.

Impression: man with severe heart disease. No evidence that his problems are related to Prevagen.

13. Pt# 40-111270; MFR# CN-120015 (2012)

58 year old man with glioblastoma multiforme, stage 4. Patient also has history of traumatic head injury and seizures. He was on Cymbalta. Glioblastoma multiforme was removed and he was started on radiation and chemotherapy. He took six 20 mg/day while in a study.

Impression: Pre-existing condition. No evidence his problems are related to Prevagen.

14. Pt# 40-108619; MFR# CN-120045 (2011)

52 year old woman with a period of hypotension reported at 44/12. She has reported previous episodes of hypotension. Also has reported insomnia. On one 40 mg Prevagen/day.

Impression: Not enough information to conclude what went on. No evidence her problem is related to Prevagen.

15. Pt# 40-114820; MFR# CN-120074

54 yr old woman with intraductal papilloma of right breast. Patient has a history of relapsing remitting Multiple Scelrosis, ankylosing spondylitis, memingioma. She takes Copaxane, Propanolol, Zolpidem, Sertraline, Gabapentin, Nitrofurantoin, daily multi vitamin. On Prevagen one 40 mg capsule per day.

Impression: Patient with a number of chronic severe diseases. Problems not likely related to Prevagen.

16. Pt#40-131071; MFR# CN-120193 (2012)

Man with history of hypertension on anti-hypertension medications. Says he had two spikes of BP after taking Prevagen. One went up to 280/180, no data on second one. No sequelae known. Took Prevagen 10 mg/day for a week before first event.

Impression: No evidence that events were related to Prevagen.

17. Pt #40-106887; MFR# 120299 (2012)

43 year old woman with relapsing multiple sclerosis, who is undergoing a relapse manifested by "blurry and double vision, falling, weakness in arms and legs, and pain". On medications Cymbalta, Gabapentin, Percocet, Baclofen, Ambien CR, Nuvigil, Ampyra. She was on 20 mg, 40 mg, and 60 mg over a period of about a year.

Impression: Pre-existing condition which explains her symptoms. Not related to Prevagen.

18. Pt# 40-056558; MFR# CN-120359 (2012)

90 year old woman found dead in her chair. Patient had risk factors for sudden death (myocardial infarction 10 years earlier, coronary artery disease treated with stenting, and cardiac valve problems). She had severe Alzheimer's. High blood pressure and ruptured intracranial aneurysm postulated for her death.

Impression: Pre-existing conditions explain her death. Not related to Prevagen.

19. Pt# CN-130077; MFR# QB-40-085112 (2011)

70 year old man who had a right hemisphere stroke, causing left homonymous hemianopsia. Patient had risk factors for stroke (atrial fibrillation and hyperlipidemia). Patient was on Lipitor, Atenolol, Aspirin. Took 10 mg Prevagen/day; not clear how long.

Impression: atrial fibrillation was most likely the cause of the stroke. No evidence that Prevagen was involved.

20. Pt# 40-106579; MFR# CN-130419 92013)

80 year old woman who "passed out". She was on metoprolol, lisinopril and pradaxa. The fact that she was on Pradaxa suggests she might have atrial fibrillation or other arrhythmia. No other information available.

Impression: Little information for conclusion, but no evidence that Prevagen was involved.

21. Pt#40-172426; MFR# CN-130478 (2013)

80 year old woman with hypercalcemia with symptoms since June 2013; taken to hospital with Calcium of 15. Took Prevagen 20 mg/day from 8/1 to 8/21/2013;

Impression: Not enough information for conclusion as to cause. No evident relation of high calcium with Prevagen.

22. Pt#40-160248; MFR# CN-130491 (2013)

85 yr old woman who had chest pain and had a "heart attack". She was hospitalized and two of her coronary arteries were "cleared out". She had family history of heart disease (father). She had been taking 20 mg/day Prevagen for one month.

Impression: no evidence the heart attack was related to Prevagen. It takes many years for coronary arteries to clog up and she had been taking Prevagen a few months.

23. Pt#40-177606; MFR#CN-130578 (2013)

85 year old woman with colitis, hypertension and heart disease. Sick to stomach and vomiting and was admitted to hospital.. Discharged apparently with the same diagnoses. She had been on 10 mg/day Prevagen.

Impression: Woman with chronic abdominal disease. Problem not likely related to Prevagen.

24. Pt#40-168283: MFR#CN-130533 (2013)

85 year old man experienced a "slight stroke". He was discharged from hospital after one day. He was taking Areda formula, Pravastatin, Isosorbide mononitrate, Doxazocin, Coumadin, Docusate calcium, vit D3, Vit e, Metoprolol, Imipramine. He was also on Prevagen 20 mg/day. No other information available.

Impression: He was taking multiple drugs for multiple chronic conditions. Unlikely Prevagen was related to his event.

25. Pt#40-073139; MFR# CN-130548 (2013)

85 year old with chest pain, irregular pulse, and elevated blood pressure. She has pre-existing hypertension and macular degeneration. Her usual BP is 160/80. She was reported to have 246/226 at hospital. Patient discharged on second day. Apparently there were no sequelae. Had been on 10 mg twice a day Prevagen for 3 weeks.

Impression: Patient with longstanding hypertension. Prevagen unlikely implicated in this SAE.

26. Pt#40-172783; MFR#CN-130530 (2013)

86 year old woman who had stroke. Gave history of heart palpitations. She was taking Prevagen 10 mg twice a day, though intermittently. She was not on other medications. No other information available.

Impression: Very little information. Unlikely that this was caused by Prevagen.

OTHER REPORTS REVIEWED:

Pt#40-121126; MFR# CN-130535 (2013)

Woman complaining of low blood pressure of below 65. Taking Prevagen for 2 years/ Patient also diabetic. Says her husband mixed up her diabetes medications.

Impression: should not be considered an Adverse event.

Pt#40-106579; MFR# CN-139419

90 yr. old woman: took green powder or green tablet (unclear which) and shortly after passed out and was hospitalized. She was subsequently released home with no evident sequelae. She also takes pradaxa (anti-coagulant) and metoprolol (B blocker); also lisinipril, ranitidine, and donepezil;

Impression: No evidence that the problem was caused by the Prevagen. It is not even clear that she was taking Prevagen at the time.

Pt#40-156568; MFR# CN-40-130423

Man reported a rise in blood pressure while taking Prevagen. Also on Crestsor, Niacin, aspirin. He normally reports low blood pressure. He stopped taking Prevagen and is fine.

Impression: I doubt this is an Adverse Event. If so, not related to Prevagen.

#QA-705-B v4

Woman suddenly died; cause unclear; had heart attack in 2002 and had stents put in, then had heart valve problems; taking depocal for mood but stopped it a few days before her death; Was also taking pregnenalone.

Impression: no evidence that death related to Prevagen.

#40-146267

Woman reported that blood pressure rose from 150 systolic to 186; long history of high blood pressure; on Prevagen; blood pressure only spiked and returned to previous readings; continued to take Prevagen; Impression: no evidence that spike in blood pressure due to Prevagen.

Pt#40-172059' MFR# CN-130463 (2013)

Complaint of blood pressure rising to 172/83. Customer has hypertension and is on a number of anti-hypertensive medications. Admits to not being very good about taking her medications. Was on Prevagen.

Impression: no evidence higher blood pressure related to Prevagen.

Pt#40-175477; MFR# CN-130595 (2013)

Woman reported high blood pressure reading: 174/99. She was on anti-hypertension medication. No further problems reported. Taking Prevagen Extra Strength 20 mg.

Impression: Chronic condition. Not related to Prevagen.

Pt#40-179420; MFR# CN-130617

Subject with hypertension and atrial fibrillation. On medication for hypertension and hypercholesterolemia. Took Prevagen for one week before episode of high BP.

Impression: Chronic conditions not related to Prevagen.

Pt#40-176927; MFR# CN-130556 (2013)

Man with diabetes and hypertension who complains of higher blood pressure while taking Prevagen. On Lopressor, Benicar, glucophage, Zocor, and Zetia plus a number of vitamins and minerals. Blood pressure better within a month.

Impression: Chronic conditions not related to Prevagen.

Conclusion:

Impression: Chronic conditions not related to Prevagen.

CONCLUSION:

After reviewing the 26 reports characterized as "serious adverse events" by Quincy and several complaints classified as "non-serious," I can state with a high degree of confidence that Prevagen was likely not the causative agent. Many of the individuals either had pre-existing conditions, some of which were complicated, and others were on several concomitant medications. As discussed above, I found no pattern of events that led me to think there was a particular safety problem with Prevagen.

(b) (6)

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June 16, 2014

Exhibit 6

Pages 000191-000312 of Curriculum Vitae removed in accordance with the Privacy Act of 1974.

SUBMISSION END