DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service
Food and Drug Administration

Memorandum

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Date: JAN 09 2008

From: Consumer Safety Officer, Division of Dietary Supplement Programs , Office of Nutritional Products, Labeling and Dietary Supplements, HFS-810

Subject: 75-Day Premarket Notification of New Dietary Ingredients

To: Dockets Management Branch, HFA-305

Subject of the Notification: Aequorin/Apoaequroin

Firm: Quincy Bioscience Manufacturing, Inc.

Date Received by FDA: September 5, 2007

90-Day Date: December 4, 2007

In accordance with the requirements of section 413(a) of the Federal Food, Drug, and Cosmetic Act, the attached 75-day premarket notification and related correspondence for the aforementioned substance should be placed on public display in docket number 95S-0316 as soon possible since it is past the 90-day date. Thank you for your assistance.

Theresa Prigmore

(95S-0316)

(RPT 424)

DEPARTMENT OF HEALTH AND HUMAN SERVICES

Public Health Service

Food and Drug Administration
5100 Paint Branch Parkway
College Park, Maryland 20740

DEC 7 2007

Mark Y. Underwood, President
Quincy Bioscience Manufacturing, Inc.
455 Science Drive, Suite 120
Madison, Wisconsin 53711

Dear Mr. Underwood:

This is to inform you that the notification, dated August 31, 2007, you submitted pursuant to 21 U.S.C. 3501b(a)(2) (section 413 of the Federal Food, Drug, and Cosmetic Act (the Act)) was received by the Food and Drug Administration (FDA) on September 5, 2007. Your notification concerns the new dietary ingredient Aequorin, which you alternately refer to as Apoaequorin that you intend to sell to manufacturers as a dietary supplement product ingredient.

According to your notice, your recommendation to dietary supplement manufacturers is to "formulate products to provide a daily dosage of 5mg aequorin. The ingredient is intended for use by persons who wish to maintain adequate levels of calcium-binding proteins in their body."

Under 21 U.S.C. 350b(a), the manufacturer or distributor of a dietary supplement containing a new dietary ingredient that has not been present in the food supply as an article used for food in a form in which the food has not been chemically altered must submit to FDA, at least 75 days before the dietary ingredient is introduced or delivered for introduction into interstate commerce, information that is the basis on which the manufacturer or distributor has concluded that a dietary supplement containing such new dietary ingredient will reasonably be expected to be safe. FDA reviews this information to determine whether it provides an adequate basis for such a conclusion. Under section 350b(a)(2), there must be a history of use or other evidence of safety establishing that the new dietary ingredient, when used under the conditions recommended or suggested in the labeling of the dietary supplement, will reasonably be expected to be safe. If this requirement is not met, the dietary supplement is considered to be adulterated under 21U.S.C. 342(f)(1)(B) because there is inadequate information to provide reasonable assurance that the new dietary ingredient does not present a significant or unreasonable risk of illness or injury.

FDA has carefully considered the information in your submission and the agency has significant concerns about the evidence on which you rely to support your conclusion that a dietary ingredient, Aequorin/Apoaequorin, will reasonably be expected to be safe. FDA was unable to determine the identity of your new dietary ingredient. The terms Aequorin and Apoaequorin refer to two distinctly different chemicals. Aequorin is a conjugated protein which consists of apoaequorin bound to coelenterazine, therefore the two terms cannot be used interchangeably.

Your notification relates the safety of your new dietary ingredient to its ingestion by people who eat jellyfish in which it occurs. However, it is difficult to rationalize the comparison of your supplement serving size with typical jellyfish consumption especially considering that you state "a typical dietary supplement serving (one capsule for example at 10-20mg) would be the equivalent of 400-800 lbs of raw jellyfish consumed."

According to your notification, your new dietary supplement ingredient is not extracted from jellyfish. It is a recombinant bacteria fermentation product. It is unclear to FDA how "aequorin/apoaequorin" is qualitatively or quantitatively similar to the material used in the studies you provide to show safety and thus, it is unclear how the information submitted is relevant the safe use of your dietary ingredient. In addition, the studies you provide do not include chronic or subchronic studies, or any demonstration of the effect of long-term consumption of your new dietary supplement ingredient.

For the reasons discussed above, the information in your submission does not provide an adequate basis to conclude that "aequorin/apoaequorin", when used under the conditions recommended or suggested in the labeling of your product, will reasonably be expected to be safe. Therefore, your product may be adulterated under 21 U.S.C. 342(f)(1)(B) as a dietary supplement that contains a new dietary ingredient for which there is inadequate information to provide reasonable assurance that such ingredient does not present a significant or unreasonable risk of illness or injury. Introduction of such a product into interstate commerce is prohibited under 21 U.S.C. 331(a) and (v).

Your notification will be kept confidential for 90 days after the filing date of September 5, 2007. After the 90-day date, the notification will be placed on public display at FDA's Division of Docket Management in docket number 95S-0316. Prior to that date, you may wish to identify in writing specifically what information you believe is proprietary, trade secret or otherwise confidential for FDA's consideration.

If you have any questions concerning this matter please contact me at (301) 436-1448.

Sincerely yours,

Linda S. Pellicore

Linda S. Pellicore, Ph.D.
Supervisory Team Leader, Senior Toxicologist
Division of Dietary Supplement Programs
Office of Nutritional Products, Labeling
and Dietary Supplements
Center for Food Safety and Applied Nutrition

SUBMITTING TO:

Attn: Dr. Linda Pellicore
Office of Nutritional Products, Labeling and Dietary Supplements
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5100 Paint Branch Parkway
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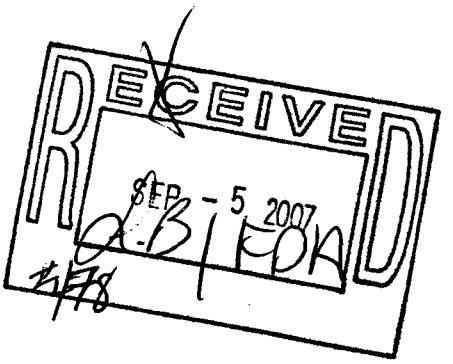

2007-1675

NEW DIETARY INGREDIENT NOTIFICATION FOR AEQUORIN

Quincy Bioscience Manufacturing, Inc.
University Research Park
455 Science Drive
Suite 120
Madison, WI 53711

Contact: Mark Y. Underwood
President
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August 31, 2007

NEW DIETARY INGREDIENT NOTIFICATION FOR AEQUORIN

Table of Contents

1. Justification

Pursuant to section 413(a)(2) of the Federal Food, Drug, and Cosmetic Act ("Act"), and FDA's implementing regulations at 21 CFR 190.6, Quincy Bioscience Manufacturing, Inc. ("Quincy") submits this New Dietary Ingredient Notification for aequorin.

2. Name and Address of the Manufacturer

Manufacturer:

Quincy Bioscience Manufacturing, Inc.
University Research Park
455 Science Drive
Suite 120
Madison, WI 53711

Principal contact and correspondent:

Mark Y. Underwood
President
Telephone: 608-233-2475
Fax: 262-364-3178

Alternate contact:

Dan Moran
Director of Manufacturing Sciences
University Research Park
455 Science Drive
Suite 120
Madison, WI 53711
Telephone: 608-233-2475
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Distributor:

Quincy Bioscience Manufacturing, Inc.
University Research Park
455 Science Drive
Suite 120
Madison, WI 53711

3. Name of the New Dietary Ingredient

The name of the new dietary ingredient is aequorin/apoaequorin. Both names are used interchangeably.

4. Applications

Aequorin is suitable as ingredient in dietary supplements, in various forms, including softgels, capsules and supplement bars and similar products. Quincy Bioscience recommends that dietary supplement manufacturers formulate products to provide a daily dosage of 5mg aequorin. The ingredient is intended for use by persons who wish to maintain adequate levels of calcium-binding proteins in their body.

5. Description of the New Dietary Ingredient

One of the first reporters used to monitor intracellular Ca2+ levels in living cells was the bioluminescent Ca2+-sensitive protein complex, aequorin (Ridgway and Ashley, 1967). Originally isolated from the luminescent jellyfish *Aequorea aequorea* (Shimomura *et al.*, 1962), the aequorin complex is made up from an apo-protein (apoaequorin) of ~21 kDa that contains four helix-loop-helix "EFhand" domains (three of which can bind to Ca2+; Head *et al.*, 2000), a prosthetic co-factor of ~420 Da (coelenterazine) and O2, in the form of peroxide (Shimomura and Johnson, 1978). In the presence of free Ca2+, the coelenterazine is oxidized to coelenteramide, with an associated release of CO2 and blue light (at ~470 nm) is produced (Shimomura and Johnson, 1973). Following this reaction, the aequorin complex is effectively "spent" and active aequorin cannot be regenerated unless additional coelenterazine is provided (Shimomura and Johnson, 1975).

In living cells, aequorin has a wide dynamic range (i.e., 0.1-100 μM Ca2+), where the light output of the aequorin luminescence reaction is approximately proportional to the square of the free Ca2+ concentration and thus it shows inherent contrast enhancement (Shimomura and Inouye, 1996). In addition, Ca2+-bound aequorin emits light without excitation, thus the autofluorescence and photo-induced cytotoxicity that can take place with fluorescent Ca2+ reporters, does not occur with aequorin. Aequorin has also been shown not to interfere with the normal development of living embryos (Miller *et al.*, 1994).

5.1. Chemistry

Aequorin is a well-known calcium-binding photoprotein found in the jellyfish, *Aequorea victorea,* that emits light by an intramolecular reaction in the presence of Ca(2+) (Shimomura et al., 1962; 1963; Shimomura and Johnson, 1978). Aequorin has been used as an intracellular Ca(2+) indicator for more than twenty-five years (Ridgeway, 1967).

Aequorin binding calcium

The protein is non-toxic when introduced into foreign cells (Blinks, 1990). Aequorin is also classified as a luminescent protein. Whenever three Ca(2+) molecules bind with one aequorin molecule, a brief flash of fluorescent light is emitted. In research applications, scientists have injected the photoprotein into thousands of cellular structures to observe the intensity of light emitted in such mechanisms in order to determine calcium concentrations, movement and signaling in various biological systems (Blinks, 1990).

Research has shown that aequorin is composed of 196 amino acids and contains three EF-hand structures of Ca(2+) binding domains (Inouye et al., 1985). The functions of these domains provide a chelation (Ca(2+)-binding) function of the protein. All of the components required for luminescence are bound tightly together and behave as a single macromolecule (Blinks, 1990) with calcium regulating the function of the protein, but not essential for it. This prepackaging is important because it means that the photoprotein can be purified, concentrated, stored and introduced into cells without any special precautions being needed to maintain proper stoichiometry of the reacting species (Blinks, 1990).

Aequorin 3-D molecule

5.2. Specifications

Conventional purification of aequorin from the jellyfish *Aequorea victoria* requires laborious extraction procedures and sometimes yields preparations that are substantially heterogeneous or that are toxic to the organisms under study.[20,21] Recombinant aequorin is safer than purified aequorin directly from jellyfish because of controlled manufacturing circumstances. Two tons of jellyfish typically yield ~125 mg of the purified photoprotein.[22] In contrast, recombinant aequorin is produced by purifying apoaequorin from genetically engineered *Escherichia coli*, followed by reconstitution of the aequorin complex *in vitro* with pure coelenterazine.[23] This method of preparation yields a pure, nontoxic, fully charged aequorin complex that is suitable for measuring intracellular Ca^{2+} by microinjection or other loading techniques.

Pressure injection has been employed to study the effects of caffeine on mouse diaphragm muscle fibers[24] and the role of Ca^{2+} in the fertilization of sea urchin eggs.[25] Alternatively, human platelets have been transiently permeabilized to the aequorin complex with DMSO,[26] and monkey kidney cells have been loaded by hypoosmotic shock.[27]

Bibliography (for above)
20. Biochem J 270, 309 (1990); **21.** J Gen Physiol 85, 189 (1985); **22.** Biochemistry 11, 1602 (1972); **23.** Biochem Biophys Res Commun 126, 1259 (1985); **24.** Neurosci Lett 127, 28 (1991); **25.** J Cell Biol 100, 1522 (1985); **26.** Biochem Biophys Res Commun 177, 888 (1991); **27.** Am J Physiol 247, C396 (1984);

(see also Material Safety Data Sheets, Appendix)

5.3. Process Description

5.3.1. General

Physical/Chemical Properties:
Physical Appearance and State: Light, fluffy, yellow-white powder.
Bibliography and Citation Index: "The crystal structure of the photoprotein aequorin at 2.3 A resolution." Head JF, Inouye S, Teranishi K, Shimomura O. Nature 405, 372-376
Property Value At Temperature or Pressure
Molecular Weight: Aequorin = 22,727.3 AMU Apoaequorin = 22,272 AMU
pH N/A
BP/BP Range N/A
MP/MP Range N/A
Freezing Point N/A
Vapor Pressure N/A
Vapor Density N/A
Saturated Vapor Conc. N/A
Bulk Density N/A

Odor Threshold N/A
Volatile% N/A
VOC Content N/A
Water Content N/A
Solvent Content N/A
Evaporation Rate N/A
Viscosity N/A
Surface Tension N/A
Partition Coefficient: U/K
Decomposition Temp. 60 C
Flash Point N/A
Explosion Limits N/A
Flammability N/A
Autoignition Temp N/A
Refractive Index N/A
Optical Rotation N/A
Solubility: Highly soluble in water.

5.3.2. Process principle

Recombinant fermentation is a well known discipline and all GMPs are followed in the manufacturing of aequorin.

5.3.3. Process outline

There has been developed a proprietary recipe for the fermentation of the aequorin which is robust and very replicable.

5.3.4. Control of manufacture

One concern with recombinant technology is of course the assurance that the natural protein has been properly expressed in the recombinant form. We have assured that this is the case by undergoing gene sequencing. (See Appendix, Apoaequorin production *E. coli* Strain QB6-#69A)

5.3.5. Purification processes

Recombinant apoaequorin expressed in the periplasmic space of *Escherichia coli* cells was regenerated into aequorin and extracted from the cells, simultaneously, using a buffer that contained coelenterazine. Due to the mild extraction conditions, the impurities in the extract were minimal. Thus, the purification of extracted aequorin could be accomplished in only two steps, anion-exchange chromatography and hydrophobic interaction chromatography, simply by adsorption and elution in both steps. The purified recombinant aequorin was pure, based on various data, including HPLC analysis and light-emitting activity. The yield of purified aequorin was 25–35 mg from 600 ml of

culture, which was over 75% of the total amount of apoaequorin expressed in *E. coli* cells.

(for more details on purification see also The *in Situ* Regeneration and Extractions of Recombinant Aequorin from *Escherichia coli* Cells and the Purification of Extracted Aequorin, Appendix)

5.3.6. Control of the end product aequorin

The finished product is routinely tested to assure it meets specifications. These are set to ensure a high degree of purity consistent with its use as a dietary supplement.

6. Occurrence in the diet

Jellyfish is a common food source comprising low fat and high protein qualities that is enjoyed around the world. Following are several references that illustrate the demand, market and history of jellyfish consumption around the globe.

- Jellyfish are commonly consumed in Thailand, Malaysia, China and Japan (Developmental Fishery Report on the Jellyfish Fishery, Appendix).
- Americans are also starting to eat jellyfish (Return of the Blob – Jellyfish as food – Light Elements, Appendix).
- Jellyfish recipes are commonly found in Chinese cookbooks sold on Amazon.com (The New Classic Chinese Cookbook, Appendix).
- Japanese aficionados enjoy jellyfish ice cream (Japans Screams for Jellyfish Ice Cream, Appendix).
- Fisherman have success in Pakistan harvesting jellyfish for consumption in China (Iran Daily Article,Appendix)

6.1. Aequorin in the diet

- International traders of jellyfish consumables conduct commerce on the internet selling frozen jellyfish heads, salted jellyfish legs as well as fresh and canned jellyfish pieces (web pages, Appendix).
- Jellyfish recipes including sesame-flavored, dried, cold-tossed and jellyfish salad are very popular in Australia (Food Down Under, Appendix)
- Both the traditional and contemporary Chinese consume jellyfish regularly as a part of their diet. (Abstracts, Appendix)

6.2. Level of consumption

The level of aequorin consumption in a typical dietary supplement serving (one capsule for example at 10-20mg) would be the equivalent of 400-800 lbs of raw jellyfish consumed.

7. Safety determination of aequorin

Acute oral toxicity study of aequorin with 14-day post-treatment observation period in rats (Limit test)

General information:
Single oral dose of 5,000 mg/kg body weight of aequorin was applied to groups of 5 male and 5 female rats by gavage. A group of 5 male and 5 female animals were also treated with the vehicle as a control. Animals were weighed, observed for lethality and toxic symptoms for 14 days. Gross pathological examination was carried out on the 15th day.

Lethality, Clinical symptoms and Body weight:
No lethality, adverse clinical symptoms, body weight loss were noted at single oral limit dose 5,000 mg/kg bw.

Gross pathology
All animals survived until the scheduled autopsy on Day 15. All organs of all male and female rats proved to be free of treatment related gross pathological changes.

Evaluation:
Single oral LD50 is higher than 5000 mg/kg bw. No adverse reaction was observed after the single oral treatment at 5000 mg/kg bw dose level in male and female Wistar rats.

8. Safety studies from literature

Aequorin has a very extensive track record of being introduced into many different cell types that were at the time being investigated with regard to how calcium was behaving within that cell type or culture. Aequorin has been tried and therefor

8.1.1. Studies with animals

Acute oral toxicity study of aequorin with 14-day post-treatment observation period in rats (Limit test)

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Evaluation:
Single oral LD50 is higher than 5000 mg/kg bw. No adverse reaction was observed after the single oral treatment at 5000 mg/kg bw dose level in male and female Wistar rats.

(See Appendix for full details of study)

8.1.2. Studies by others with products containing aequorin.

What Safety Testing Has Been Done?
All BioToy ingredients have been tested for skin, eye and oral (eaten) toxicity, as well as specialized testing of the NanoFuel chemical to make sure it cannot damage DNA, and thus be a potentially cancer causing chemical. Our testing has been done by our own scientists as well as two independent testing laboratories. We summarize the results here, but upon request we can supply complete copies of the test reports:

1. ACTS Testing Laboratories, Buffalo, New York. (independent laboratory)

NanoLight, NanoFlash, and NanoFuel have all been tested for acute oral toxicity, skin irritation, and eye irritation potential. The compounds were not considered to be toxic, and were not considered to be primary skin irritants or eye irritants as defined in the Federal Hazardous Substances Act Regulations, Title 16, Code of Federal Regulations, Section 1500.3.

2. Innova Biomed Irvington, New York. (BioToy scientists)

NanoFuel was tested for subactute oral toxicity in rats over a fourteen day treatment period. There was no significant toxicity noted. NanoFuel was tested for subacute toxicity in mice by injection over a fourteen day treatment period. There was no significant toxicity noted. NanoFuel was tested for eye irritation and skin irritation in rabbits. No significant skin or eye irritation was noted in any of the animals tested.

3. Quintiles (independent laboratory)

NanoFuel was subjected to Ames testing. This is a series of tests designed to determine whether or not a chemical substance can cause a mutation in various bacterial strains. If

the chemical tested can mutate a bacterium, it can be considered a potential human carcinogen. BioToy NanoFuel was negative for mutagenicity in the Ames test.

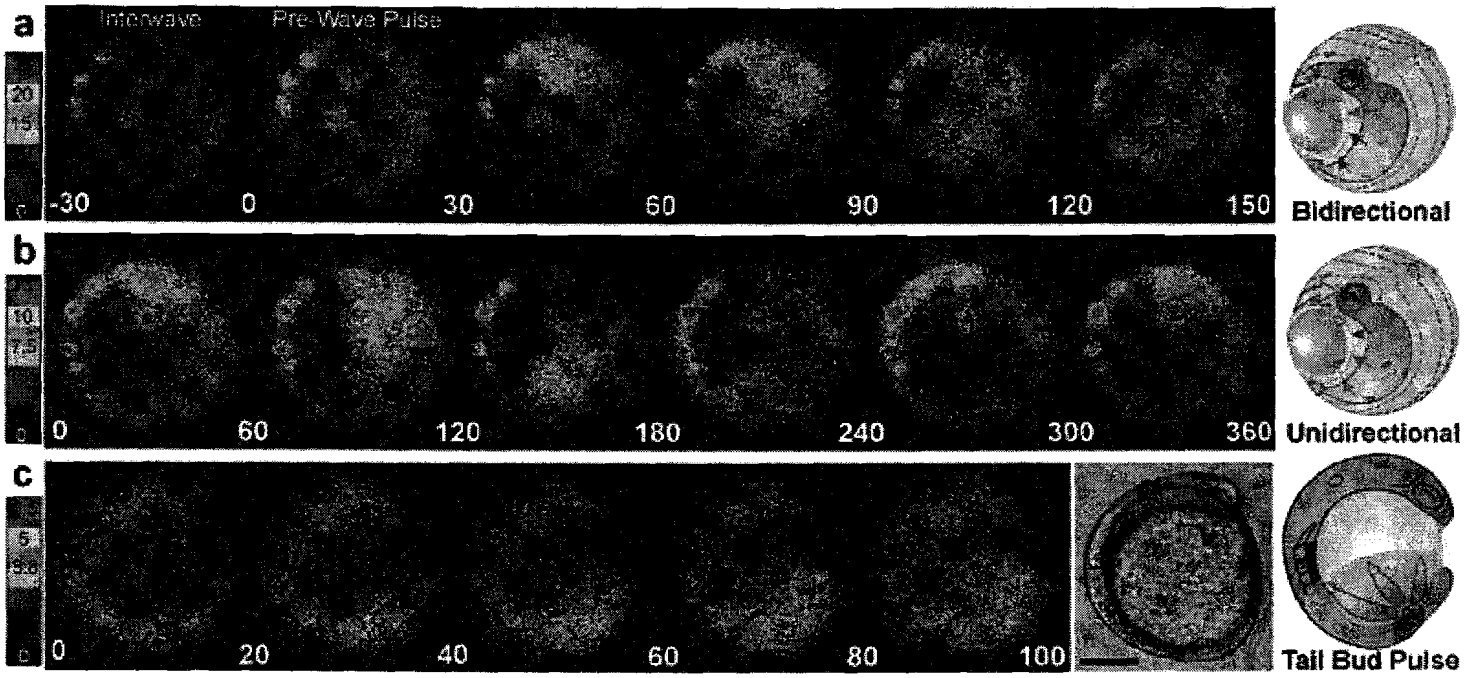
8.1.3. Introduction of aequorin in cellular structures

Aside from the fluorescent antibody techniques having few practical applications for intracellular imaging in living cells,[1] the most significant advances came from the development of small organic fluorescent molecules by Roger Tsien's laboratory that were responsive to ionic signals, such as Ca^{2+}, H^{+}, and membrane potential ($\Delta\Psi_m$), and could be introduced into live cells, generally without significant toxicity.[2] Many of

8.1.4. Studies on embryos

Aequorin has also been shown not to interfere with the normal development of living embryos (Miller *et al.*, 1994). Aequorin has a number of advantages over other Ca2+ indicators, for example, low leakage rate from cells, lack of intracellular compartmentalization or sequestration and it does not disrupt cell functions or embryo development. (Nanolight MSDS, in Appendix)

Figure 19.65 Images of Ca^{2+} waves in gastrulating zebrafish embryos detected by microinjected *f*-aequorin (recombinant aequorin reconstituted with the coelenterazine *f* luminophore (C6779)). The images are pseudocolored to represent Ca^{2+}-dependent luminescent flux in (photons/pixel/second $\times 10^{-2}$) according to the color scales shown at the left of each of the three time-lapse image sequences (a,b,c). Time in seconds is indicated in the lower left-hand corner of each frame. The sequences depict three different spatial wave types that are represented schematically at the end of each sequence. **PM** indicates the dorsal midline pacemaker; its position in the luminescence images is marked by a red asterisk. The image was contributed by Edwin Gilland, Marine Biological Laboratory, Woods Hole, MA, and reproduced with permission from Proc Natl Acad Sci U S A 96, 157 (1999).



9. Discussion and Conclusions

V. Signature of Designated Person

8/8/07

Mark Y. Underwood
President

10. References

MILLER, A.L., KARPLUS, E. and JAFFE, L.F. (1994). Imaging [Ca2+]i with aequorin using a photon imaging detector, In *Methods in Cell Biology* (R. Nuccitelli, Ed.) Vol. 40, pp. 305-338. Academic Press, San Diego, CA.

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SHIMOMURA, O. and JOHNSON, F.H. (1978). Peroxidized coelenterazine, the active group in the photoprotein aequorin. *Proc. Natl. Acad. Sci. USA* 75: 2611-2615.

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SHIMOMURA, O. and JOHNSON, F.H. (1975). Regeneration of the photoprotein aequorin. *Nature* 256: 236-238.

Appendices

Molecular Probes AquaLite Recombinant Aequorin – Material Safety Data Sheet

Prolume Aequorin/Apoaequorin Material Safety Data Sheet

The *in Situ* Regeneration and Extractions of Recombinant Aequorin from *Escherichia coli* Cells and the Purification of Extracted Aequorin

NanoLight – Material Safety Data Sheet

Developmental Fishery Report on the Jellyfish Fishery

Return of the Blob – Jellyfish as food – Light Elements

The New Classic Chinese Cookbook

Japans Screams for Jellyfish Ice Cream

Iran Daily Article

Food Down Under

Web pages

Abstracts