

GRAS Notice (GRN) No. 519

<http://www.fda.gov/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/default.htm>

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GRN 000519

May 8, 2014

Paulette M. Gaynor, Ph.D.
Deputy Division Director
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

RE: Whole *Chlorella protothecoides* S106 Algal Protein GRAS Notification

Dear Dr. Gaynor:

In accordance with proposed 21 CFR § 170.36 (notice of a claim for exemption based on a GRAS determination) published in the Federal Register (62 FR 18937-18964), I am submitting, as the agent of the notifier, Solazyme, Inc., 225 Gateway Blvd. South San Francisco, CA 94080 a GRAS notification for the use of whole *Chlorella protothecoides* S106 algal protein as a dietary protein, analogous to soy- and animal-based proteins, in the diet at a maximum consumption level of 5562 mg/day. A GRAS expert panel dossier, setting forth the basis for the GRAS determination, as well as *curriculum vitae* of the members of the GRAS panel, are enclosed.

Best regards,

(b) (6)

Nancy J. Szabo, Ph.D.
Senior Toxicologist



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1. GRAS Exemption Claim

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

Solazyme, Inc. has determined whole *Chlorella protothecoides* S106 algal protein, derived from a non-toxicogenic strain of *Chlorella protothecoides*, to be generally recognized as safe (GRAS) as a food ingredient and therefore, exempt from the requirement of premarket approval and from environmental impact, under the conditions of its intended use as described below. The common name for this ingredient is Whole Algal Protein (WAP). WAP is to be used as a protein source, analogous to soy- and animal-based proteins, in a variety of conventional foods,¹ none of which have a standard of identity.² The basis for this finding is described in the following sections.

Signed,

(b) (6)

Date 09 MAY 2014

Nancy J. Szabo, Ph.D.
Senior Toxicologist
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¹ Baked goods and mixes, breakfast cereals, meal replacements, cheeses, milk products, dairy and nondairy products, egg products, fish products, meat products, poultry products, plant protein products, grain products and pastas, gravies and sauces, salad dressings, margarines, processed vegetables and vegetable juices, fresh and processed fruit juices, nonalcoholic beverages, gelatins and puddings, frozen dairy, soups, nut products, snack foods and soft candy.

² All food categories designated by Solazyme have been utilized in the estimated dietary intake calculations as appropriate; however, certain categories designated by Solazyme may contain foods for which a standard of identity exists. We note that an ingredient that is lawfully added to food products may be used in a standardized food only if it is permitted by the applicable standard of identity. Solazyme confirms that WAP will be added only to foods for which a standard of identity does not exist.

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(i) Name and Address of the Notifier

Solazyme, Inc.
225 Gateway Blvd
South San Francisco, CA 94080

Agent of the Notifier:

Nancy J. Szabo, Ph.D.
Senior Toxicologist
Burdock Group
859 Outer Road
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Telephone: 407-802-1400
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(ii) Common Name of the Notified Substance

The common name of whole *Chlorella protothecoides* S106 algal protein for the purposes of this GRAS Notification has been defined as:

Whole Algal Protein (WAP)*

* Synonyms: Algal powder, Algal protein, Algalin protein, Whole algalin protein

(iii) Conditions of Use

WAP may be used as an ingredient in the food groups shown in Appendix 1 in order to provide dietary protein, analogous to soy- and animal-base proteins, for consumption levels up to 5562 mg *per* day, by individuals who desire an increase of non-animal-based protein and/or a reduction of animal-based protein in their diet.

(iv) Basis of GRAS Determination

Pursuant to 21 CFR § 170.3, the use of WAP as an ingredient in food categories shown in Appendix 1, at an intended maximum 90th percentile consumption of 5.56 g *per* day, has been determined GRAS by scientific procedures for its intended conditions of use. The safety of WAP for this use is supported by publicly available information including, but not limited to, a 13-week dietary toxicity study in the rat and two genotoxicity studies (*in vitro* bacteria reverse mutation assay and *in vivo* chromosome aberration assays) (Szabo *et al.*, 2013), as well as a 28-day repeated dose study on the similarly composed ground yellow, high-lipid *C. protothecoides* biomass (Day *et al.*, 2009; FDA, 2012) and a 13-week dietary toxicity study in the rat and two genotoxicity studies (*in vitro* bacteria reverse mutation assay and *in vivo* chromosome aberration assays) on the closely related High Lipid Algalin Flour (HLAF) from dried milled *C. protothecoides* (Szabo *et al.*, 2012; FDA, 2013). This determination is based on the views of

experts who are qualified by scientific training and experience to evaluate the safety of substances used as ingredients in food.

(v) Availability of Information

The data and information (*i.e.*, published articles, unpublished reports and referenced communications) that serve as a basis for this GRAS determination are available for FDA review and copying during conventional office hours at:

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Alternatively, data and information that serve as a basis for this GRAS determination may be sent to FDA upon request.

2. Detailed Information about the Identity of the Notified Substance

A. Identity

WAP is a pale yellow to green³ high protein powder composed of the dried biomass of *C. protothecoides* S106. The general descriptive characteristics of WAP are presented in Table 1.

Table 1. General description of WAP

Appearance	Pale yellow to green powder/flake
Packaging	Food grade, poly-lined, 3-ply heat-sealed Kraft bags (15-25 kg)
Storage	Closed package in a cool, dry place (< 25 °C)
Stability	12 months
Intended use	Dietary protein, analogous to soy- and animal-based proteins
Functionality in food	Source of macronutrients as a dietary protein, analogous to soy- and animal-based proteins

WAP = Whole Algal Powder

As a species, *C. protothecoides* is currently assigned to the genus *Chlorella* in the phylum Chlorophyta. Widespread in fresh and salt water, soil and air (Wu *et al.*, 2001), the green microalgae *Chlorella spp.* are nonmotile, unicellular eukaryotes that are spherical in shape and typically have diameters from 2 – 10 µm (Kay, 1991; Becker, 2007). The cell walls are hemicellulosic, rigid, and account for approximately 10% of the algal dry weight (Becker, 2007).

³ WAP is not added to food with the intention of acting as a color. Although WAP (which is naturally light yellow to green) can modify the color of pale foods in the same way that adding chocolate or tomatoes can alter color, the ingredient will be used in such a way that any color imparted is clearly unimportant insofar as the appearance, value, marketability or consumer acceptability is concerned in bulk amounts. WAP is, therefore, exempt from the definition of a color additive and from FDA premarket approval requirements for color additives [FFDCA §201(t) and 21 CFR§70.3(g)].

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Chlorella spp. also characteristically have membrane-bound organelles (Kay, 1991). Axenic⁴ cultures of *Chlorella* spp. are easily established, in part because replication under optimum conditions tends to be rapid, often requiring less than two hours (Kay, 1991). Protein and lipid content and the fatty acid profile are known to vary widely with the species, the stage of cell growth (exponential or stationary), and with environmental conditions (e.g., available nutrients, temperature, and light intensity) (Kay, 1991). The protein contents of *Chlorella* spp. have been reported to range from 15 – 60 % on a dry weight basis (Kay, 1991; Becker, 2007).

Although the strains are all unicellular and morphologically similar, the species grouped into the genus *Chlorella* have recently been recognized to be phylogenetically divergent (Ferris *et al.*, 2005) and not necessarily related (Tiberg and Einarsson, 1989; Huss *et al.*, 1999). Nomenclature and taxonomic assignments based on traditional identification methods are being re-examined – and when necessary, reclassified – using data from genetic sequence analysis (Ferris *et al.*, 2005). Because species comprising the *Chlorella* genus are a taxonomically complicated group, chemotaxonomic character, DNA⁵ base composition and DNA/DNA hybridization are all used to distinguish the morphologically similar species from one another (Huss *et al.*, 1999; Ferris *et al.*, 2005). For example, based on comparative analyses of 18S ribosomal RNA gene sequences, Huss *et al.* (1999) has recommended that only four species remain in the *Chlorella* genus: *C. vulgaris*, *C. lobophora*, *C. sorokiniana*, and *C. kessleri*.

Solazyme obtained *C. protothecoides* UTEX 250 from the University of Texas (UTEX) Culture Collection of Algae in Austin, Texas and assigned the Solazyme internal strain number “S106”. UTEX 250 had, in turn, been obtained by UTEX as *C. protothecoides* CCAP211/7C from the Culture Collection of Algae and Protozoa (CCAP), Scottish Association for Marine Science, Argyll Scotland, United Kingdom, where the strain was originally deposited sometime between 1952 and 1955 by A.J. Kluyver⁶ as the third isolate of *C. vulgaris* (“3 *C. vulgaris*”) taken from freshwater in Delft, Netherlands. Other deposits of this strain include the Meyer 34 and SAG211-7C accessions in the Sammlung von Algenkulturen Gottingen (SAG, Albrecht-von-Haller-Institute for Plant Science, University of Göttingen, Göttingen, Germany).⁷ Additional designations by which this strain is known include *C. protothecoides* var. *communis* Shihira & Krauss (1965),⁸ *C. protothecoides* Krüger, and *Auxenochlorella protothecoides* (Krüger) Kalina & Puncochárová (1987). Based on accepted designations, UTEX 250 (S106) may be appropriately considered a strain of *C. protothecoides* or *Auxenochlorella protothecoides*. All *C. protothecoides* (*A. protothecoides*) strains held at the American Type Culture Collection (ATCC, Rockville, Maryland) are preserved under Biosafety Level (BSL) 1 conditions which indicate the microorganisms are not recognized to cause disease in immunocompetent adult humans.⁹

The ecophysiological and biochemical characteristics of *C. protothecoides* include the inability to utilize nitrate as a substrate, the need for thiamine but not for vitamin B₁₂, the lack of secondary carotenoids (most strains) and a tolerance for high temperatures (28 – 34 °C upper

⁴ Cultures containing a single strain (*i.e.*, not contaminated by or associated with other microorganisms).

⁵ DNA = deoxyribonucleic acid

⁶ <<http://web.biosci.utexas.edu/utex/algaeDetail.aspx?algaeID=2779>>; site accessed March 31, 2014.

⁷ <http://sagdb.uni-goettingen.de/detailedList.php?str_number=211-7c>; site accessed March 31, 2014.

⁸ <<http://web.biosci.utexas.edu/utex/algaeDetail.aspx?algaeID=2779>>; site accessed March 31, 2014.

⁹ <http://www.atcc.org/Search_Results.aspx?dsNav=Ntk:PrimarySearch%7cchlorella+protothecoides%7c3%7c,Ny:True,Ro:0,N:1000552&searchTerms=chlorella+protothecoides&redir=1>; site accessed March 31, 2014.

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limits), acidic conditions (3.5 – 4.0 pH) and high salt concentrations (3 – 4%) (Huss *et al.*, 2002). Morphology for the species includes the absence of pyrenoids, but the presence of a tri-laminar layer within the cell wall (Huss *et al.*, 2002). Reproduction is by endospore, an asexual internal process through which two to twelve sporangiospores are produced and released by each parental sporangium during vegetative growth (Ramírez-Romero *et al.*, 2010). Under nitrogen depleted conditions, the cell begins to synthesize and store copious amounts of oil (Day *et al.*, 2009). When glucose is available as a carbon source and inorganic nitrogen is not restricted, the cells produce high levels of protein (Day *et al.*, 2009).

Although similar in appearance, morphology, and composition to other *Chlorella* species, such as *C. vulgaris* and *C. pyrenoidosa*¹⁰ (Robinson and Guzman-Juarez, 1978; Kay, 1991; Brown and Jeffery, 1992; Tokusoglu and Unal, 2003; Ravishankar *et al.*, 2006; Day *et al.*, 2009), *C. protothecoides* alone has the ability, when exposed to glucose, to etiolize (*i.e.*, ‘de-green’). Genetically, *C. protothecoides* strains (with the single exception of *C. protothecoides* var *Acidicola*¹¹) have been shown to be closely related to species in the achlorophyllous genus *Prototheca*. Gene-based phylogenetic trees developed from nuclear 18S rRNA¹², plastid 16S rRNA, SSU¹³ and LSU¹⁴ rDNA¹⁵, and 26S rDNA domain sequences have repeatedly demonstrated that *A. protothecoides* UTEX 25 (S485), the type strain for *A. protothecoides*, is closely related to the *Prototheca* species (Huss *et al.*, 1999; Tartar *et al.*, 2003; Ueno *et al.*, 2005; Satoh *et al.*, 2010). At least one protein-based phylogenetic tree also confirms these assessments (von Bergen *et al.*, 2009). The relationship is sufficiently homologous that comparative analysis of the phylogeny of the related *A. protothecoides* UTEX 25 (S485) and twelve strains of *Prototheca* were found to form a monophyletic clade (Ueno *et al.*, 2005).

Corroborative Genotypic Information

Delineation of microalgal species based on morphology is problematic due to potentially strong morphological and physiological similarities among genetically diverse species. Molecular indicators such as plastidic 23S rDNA sequence homology are more reliable indicators of strain relatedness. As an example, the plastid 23S rDNA sequences shown in Figure 1 demonstrate that *C. protothecoides* S106 (UTEX 250 (Solazyme source); also known as (aka) SAG 211-7C and CCAP 211/7C; original deposition CCAP(1952-5)) possesses a genotype identical (*i.e.* having 100% homology) to that of Solazyme designated strain *C. protothecoides* S485 (UTEX 25 (Solazyme source); aka, SAG 211-7A, CCAP211/7A, and ATCC 30407). Strain S485 is known formally as *Auxenochlorella protothecoides* (Krüger) Kalina & Puncochárová (1987) and is the type strain for *A. protothecoides*. *C. protothecoides* S106 is also shown to share approximately 87.5% homology with another *C. protothecoides* strain, S102 (UTEX B 25 (Solazyme source); aka original deposition CCAP (1952-5)) which is called *C. protothecoides* Krüger (1892) and *A. protothecoides*. In summary, based on plastid 23S rDNA sequencing, *C.*

¹⁰ Many of these stains have been reclassified as *C. sorokiniana* or *C. fusca* var. *vacuolata*.

¹¹ Analysis of 18S RNA gene sequences has demonstrated only a weak phylogenetic relationship to *C. protothecoides* var. *acidicola* which is no longer considered a variety of *C. protothecoides* (Huss *et al.*, 2002; Ferris *et al.*, 2005).

¹² rRNA = ribosomal ribonucleic acid

¹³ SSU = small subunit of ribulose bis-phosphate carboxylase-oxygenase

¹⁴ LSU = large subunit of ribulose bis-phosphate carboxylase-oxygenase

¹⁵ rDNA = ribosomal deoxyribonucleic acid

protothecoides S106 is identical (*i.e.* 100% homology) to *A. protothecoides* S485 (type strain of *Auxenochlorella protothecoides*) and only somewhat closely related (approximately 87.5% homology) to *C. protothecoides* S102.

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S102 23S TGTTGAAGAATGAGCCGGCGACTTAGAAAACGTGGCAAGGTTAAGGAAACGTATCCGGAG
 S106 23S TGTTGAAGAATGAGCCGGCGACTTAGAAAAAGTGGCGTGGTTAAGGAAAAAT-TCCGAAG
 S485 23S TGTTGAAGAATGAGCCGGCGACTTAGAAAAAGTGGCGTGGTTAAGGAAAAAT-TCCGAAG

 S102 23S CCGAAGCGAAAGCAAGTCTGAACAGGGCG-----ATTAAGTCA
 S106 23S CCTTAGCGAAAGCGAGTCTGAATAGGGCGATCAAATATTTTAATATTTACAATTTAGTCA
 S485 23S CCTTAGCGAAAGCGAGTCTGAATAGGGCGATCAAATATTTTAATATTTACAATTTAGTCA

 S102 23S TTTTTTCTAGACCCGAACCCGGGTGATCTAACCATGACCAGGATGAAGCTTGGGTGACAC
 S106 23S TTTTTTCTAGACCCGAACCCGGGTGATCTAACCATGACCAGGATGAAACTTGGGTGATAC
 S485 23S TTTTTTCTAGACCCGAACCCGGGTGATCTAACCATGACCAGGATGAAACTTGGGTGATAC

 S102 23S CAAGTGAAGGTCCGAACCGACCGATGTTGAAAAATCGGCGGATGAGTTGTGGTTAGCGGT
 S106 23S CAAGTGAAGGTCCGAACCGACCGATGTTGAAAAATCGGCGGATGAGTTGTGGTTAGCGGT
 S485 23S CAAGTGAAGGTCCGAACCGACCGATGTTGAAAAATCGGCGGATGAGTTGTGGTTAGCGGT

 S102 23S GAAATACCAGTCGAACTCGGAGCTAGCTGGTTCTCCCCGAAATGCGTTGAGGCGCAGCGG
 S106 23S GAAATACCAGTCGAACTCGGAGCTAGCTGGTTCTCCCCGAAATGCGTTGAGGCGCAGCAG
 S485 23S GAAATACCAGTCGAACTCGGAGCTAGCTGGTTCTCCCCGAAATGCGTTGAGGCGCAGCAG

 S102 23S TTCATA-AGGCTGTCTAGGGGTAAAGCACTGTTTCGGTGCGGGCTGCGAAAGCGGTACCA
 S106 23S TACATCTAGTCTATCTAGGGGTAAAGCACTGTTTCGGTGCGGGCTGTGAAAACGGTACCA
 S485 23S TACATCTAGTCTATCTAGGGGTAAAGCACTGTTTCGGTGCGGGCTGTGAAAACGGTACCA

 S102 23S AATCGTGGCAAACCTCTGAATACTAGATATG-CTATTTATGGGCCAGTGAGACGGTGGGGG
 S106 23S AATCGTGGCAAACCTCTGAATACTAGAAAATGACGGTGTA-GT---AGTGAGACTGTGGGGG
 S485 23S AATCGTGGCAAACCTCTGAATACTAGAAAATGACGGTGTA-GT---AGTGAGACTGTGGGGG

 S102 23S ATAAGCTTCATCGTTCGAGAGGGAAACAGCCCAGATCACTAGCTAAGGCCCCAAAATGATC
 S106 23S ATAAGCTCCATTGTCAAGAGGGAAACAGCCCAGACCACCAGCTAAGGCCCCAAAATGGTA
 S485 23S ATAAGCTCCATTGTCAAGAGGGAAACAGCCCAGACCACCAGCTAAGGCCCCAAAATGGTA

 S102 23S GTTAAGTGACAAAGGAGGTGAGAATGCAGAAACAACCAGGAGGTTTGCTTAGAAGCAGCC
 S106 23S ATGTAGTGACAAAGGAGGTGAAAATGCAAAACAACCAGGAGGTTGGCTTAGAAGCAGCC
 S485 23S ATGTAGTGACAAAGGAGGTGAAAATGCAAAACAACCAGGAGGTTGGCTTAGAAGCAGCC

 S102 23S ACCCTTTAAAGAGTGCGTAATAGCTCACTG
 S106 23S ATCCTTTAAAGAGTGCGTAATAGCTCACTG
 S485 23S ATCCTTTAAAGAGTGCGTAATAGCTCACTG

Figure 1. Plastid 23S rDNA sequences for Solazyme designated *C. protothecoides* strains S102, S106 (Solazyme strain of interest) and S485 (*Auxenochlorella protothecoides* type strain)

In similar fashion, Solazyme's *C. protothecoides* S106 strain was compared using partial plastidic 23S rDNA sequencing against 21 *C. vulgaris* strains obtained from five different depository collections. As indicated below, each test strain is identified by its Solazyme designation, common name and strain source; identical strains and other current or previous names are included as parenthetical information for ease of reference:

- (1) S183: *C. vulgaris*, UTEX 395 (formerly *C. pyrenoidosa* (Starr and Zeikus 1987));

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- (2) S184: *C. vulgaris* (type strain), UTEX 259, (CCAP 211/11B; aka *Chlorella candida*; *Chlorella miniata*; *Chlorella vulgaris* var. *vulgaris* f. *vulgaris*; original deposition CCAP 1952-5);
- (3) S185: *C. vulgaris*, UTEX 1809 (formerly *C. salina* (Starr and Zeikus 1987));
- (4) S187: *C. vulgaris*, UTEX 2714 (*Chlorella vulgaris* Bashan);
- (5) S188: *C. vulgaris*, UTEX 30 (CCAP 211/12; SAG 211-12; ATCC 16487; aka *C. vulgaris* Beijerinck f. *viridis* ((Chodat) Fott and Nováková));
- (6) S190: *C. vulgaris*, UTEX 396 (formerly *C. vulgaris* var. *viridis* (Starr and Zeikus 1987));
- (7) S191: *C. vulgaris*, UTEX 26 (formerly *Chlorella pyrenoidosa* (Starr & Zeikus 1987); aka *C. photophila* (Shihira & Krauss 1965); *C. emersonii*; *C. fusca* var. *vacuolata*);
- (8) S192: *C. vulgaris*, UTEX 265 (CCAP 211/11J; SAG 211-11J; *Chlorella vulgaris* Beijerinck 1890 (Rodhe); aka *C. simplex* (Shihira & Krauss 1965) and *Chlorella vulgaris* var. *vulgaris* f. *vulgaris*; original designation CCAP 1630);
- (9) S246: *C. vulgaris*, SAG 30.80 (*Chlorella vulgaris* Beijerinck (Senger 1965); formerly *C. saccharophila*);
- (10) S257: *C. vulgaris*, SAG 211.11T (*Chlorella vulgaris* Beijerinck (Ruschmann));
- (11) S300: *C. vulgaris*, CCAP 211/19 (*Chlorella vulgaris* Beijerinck 1890 (von Witsch 1946/7));
- (12) S301: *C. vulgaris*, CCAP 211/11S (*Chlorella vulgaris* Beijerinck 1890 (Pirson));
- (13) S302: *C. vulgaris*, CCAP 211/11Q (*Chlorella vulgaris* Beijerinck (Czurda));
- (14) S303: *C. vulgaris*, CCAP 211/11P (SAG 211-11P; *Chlorella vulgaris* Beijerinck (Algéus 1942 Strain B);
- (15) S344: *C. vulgaris*, CCAP 211/81 (*Chlorella vulgaris* Beijerinck 1890 (Krienitz 1979); original designation CCAP A36);
- (16) S345: *C. vulgaris*, CCAP 211/80 (*Chlorella vulgaris* Beijerinck 1890 (Krienitz 1979); original designation CCAP A35);
- (17) S410: *C. vulgaris*, CCALE¹⁶ 263 (*C. vulgaris* Beijerinck (Hindak 1967/61));
- (18) S412: *C. vulgaris*, CCALE 266 (*C. vulgaris* Beijerinck (Marsalek 1985/57));
- (19) S413: *C. vulgaris*, CCALE 268 (*C. vulgaris* Beijerinck (Gaffron/Bethesda C 1.3.1.));
- (20) S497: *C. vulgaris*, CAUP¹⁷ H1993 (*C. vulgaris* Beijerinck (Punčochářová 1981/22)); and

¹⁶ Culture Collection of Autotrophic Organisms (CCALA), Institute of Botany, Academy of Sciences of the Czech Republic, Třeboň, Czech Republic; <<http://ccala.butbn.cas.cz/en/type-classis-order/algae>>; site accessed March 31, 2014.

¹⁷ Culture Collection of Algae of Charles University in Prague (CAUP), Praha, Czech Republic; <<http://botany.natur.cuni.cz/algo/caup-list.html>>; site accessed March 31, 2014.

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(21) S498: *C. vulgaris*, CAUP H1996 (*C. vulgaris* Beijerinck f. *globosa* (Andreeva 1961); *C. vulgaris* var. *vulgaris* f. *globosa*).

Distance values (D values; a numerical metric used to indicate genetic relatedness) were generated from the sequence data; lower D values indicate sequences that are more closely related with '0.000' indicating identical sequences (*i.e.*, 100% homology). Higher values, on the other hand, indicate more divergent sequences. Isolates with minor genetic variation (high homology) are generally accepted as varying by a value of only about 0.005 (Rawat, *et al.*, 2005). For perspective, strains having less than 95.5% homology (based on 16S rRNA sequence identity) are routinely regarded as different species (Stackebrandt and Goebel, 1994). Based on an evaluation of the numeric metric used to assess relatedness (Table 2), the partial plastidic 23S rDNA sequence for Solazyme strain S106, the *C. protothecoides* strain of interest, had D values ≥ 0.080 relative to the 21 *C. vulgaris* strains examined. To place this in context, when the D values for Solazyme strain S184 (the type strain for *C. vulgaris*) are considered, S184 is seen to be (1) identical to 15 of the other 21 *C. vulgaris* strains tested (D value = 0.000 for all),¹⁸ (2) very closely or reasonably closely matched to three strains (D values from 0.002 to 0.034),¹⁹ and (3) more distantly related to the remaining three strains (D values ≥ 0.090).²⁰ S106 is counted among this last group, that is, it is more distantly related to the type strain for *C. vulgaris*. An additional comparison of the 23S sequence of *C. protothecoides* S106 using nBLAST analysis to that of GenBank accession No.²¹ L43357 *C. vulgaris* Beijerinck (NIES²²-2170, formerly in the IAM²³ collection as C-27; formerly classified as *C. ellipsoidea* Gerneck) determines homology of 87% (FDA, 2012). In summary, *C. protothecoides* S106 is distantly related to many of the *C. vulgaris* strains tested, including S184 (UTEX 259), but it is also notable that the *C. vulgaris* type strain S184 is more closely related to S106 than to some *C. vulgaris* isolates (*e.g.*, S412 and S498).

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¹⁸ S183, S185, S188, S190, S192, S246, S257, S300, S302, S303, S344, S345, S410, S413, and S497

¹⁹ S187, S197, and S301

²⁰ S106, S412, and S498

²¹ No. = number

²² National Institute for Environmental Studies, Tsukuba, Japan.

²³ Institute of Applied Microbiology, Center for Cellular and Molecular Research, Institute of Molecular and Cellular Biosciences University of Tokyo, Tokyo, Japan.

Table 2. Tabulated Distance values for 23S rDNA sequences of *C. protothecoides* S106 (Solazyme strain of interest) compared to S183, S184 (*C. vulgaris* type strain), S185, S187, S188, S190, S191, S192, S246, S257, S300, S301, S302, S303, S344, S345, S410, S412, S413, S497, and S498. (Lower values indicate sequences that are more closely related, with '0.000' indicating identical sequences. Higher values indicate more divergent sequences.)

	S106 23S	S183 23S	S184 23S	S185 23S	S187 23S	S188 23S	S190 23S	S191 23S	S192 23S	S246 23S	S257 23S	S300 23S	S301 23S	S302 23S	S303 23S	S344 23S	S345 23S	S410 23S	S412 23S	S413 23S	S497 23S	S498 23S	
S106 23S		0.090	0.090	0.090	0.060	0.090	0.090	0.095	0.090	0.090	0.090	0.090	0.082	0.090	0.092	0.090	0.090	0.090	0.150	0.090	0.090	0.186	
S183 23S	0.090		0.000	0.000	0.030	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.034	0.000	0.000	0.000	0.000	0.000	0.106	0.000	0.000	0.152	
S184 23S	0.090	0.000		0.000	0.030	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.034	0.000	0.000	0.000	0.000	0.000	0.106	0.000	0.000	0.152	
S185 23S	0.090	0.000	0.000		0.030	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.034	0.000	0.000	0.000	0.000	0.000	0.106	0.000	0.000	0.152	
S187 23S	0.080	0.030	0.030	0.030		0.030	0.030	0.033	0.030	0.030	0.030	0.030	0.006	0.030	0.031	0.030	0.030	0.030	0.110	0.030	0.030	0.148	
S188 23S	0.090	0.000	0.000	0.000	0.030		0.000	0.002	0.000	0.000	0.000	0.000	0.034	0.000	0.000	0.000	0.000	0.000	0.106	0.000	0.000	0.152	
S190 23S	0.090	0.000	0.000	0.000	0.030	0.000		0.002	0.000	0.000	0.000	0.000	0.034	0.000	0.000	0.000	0.000	0.000	0.106	0.000	0.000	0.152	
S191 23S	0.095	0.002	0.002	0.002	0.033	0.002	0.002		0.002	0.002	0.002	0.002	0.036	0.002	0.002	0.002	0.002	0.002	0.106	0.002	0.002	0.154	
S192 23S	0.090	0.000	0.000	0.000	0.030	0.000	0.000	0.002		0.000	0.000	0.000	0.034	0.000	0.000	0.000	0.000	0.000	0.106	0.000	0.000	0.152	
S246 23S	0.090	0.000	0.000	0.000	0.030	0.000	0.000	0.002	0.000		0.000	0.000	0.034	0.000	0.000	0.000	0.000	0.000	0.106	0.000	0.000	0.152	
S257 23S	0.090	0.000	0.000	0.000	0.030	0.000	0.000	0.002	0.000	0.000		0.000	0.034	0.000	0.000	0.000	0.000	0.000	0.106	0.000	0.000	0.152	
S300 23S	0.090	0.000	0.000	0.000	0.030	0.000	0.000	0.002	0.000	0.000	0.000		0.034	0.000	0.000	0.000	0.000	0.000	0.106	0.000	0.000	0.152	
S301 23S	0.082	0.034	0.034	0.034	0.006	0.034	0.034	0.036	0.034	0.034	0.034	0.034		0.034	0.034	0.034	0.034	0.034	0.112	0.034	0.034	0.150	
S302 23S	0.090	0.000	0.000	0.000	0.030	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.034		0.000	0.000	0.000	0.000	0.106	0.000	0.000	0.152	
S303 23S	0.092	0.000	0.000	0.000	0.031	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.034	0.000		0.000	0.000	0.000	0.109	0.000	0.000	0.156	
S344 23S	0.090	0.000	0.000	0.000	0.030	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.034	0.000	0.000		0.000	0.000	0.106	0.000	0.000	0.152	
S345 23S	0.090	0.000	0.000	0.000	0.030	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.034	0.000	0.000	0.000		0.000	0.106	0.000	0.000	0.152	
S410 23S	0.090	0.000	0.000	0.000	0.030	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.034	0.000	0.000	0.000	0.000		0.106	0.000	0.000	0.152	
S412 23S	0.150	0.106	0.106	0.106	0.110	0.106	0.106	0.106	0.106	0.106	0.106	0.106	0.112	0.106	0.109	0.106	0.106	0.106	0.106		0.106	0.106	0.123
S413 23S	0.090	0.000	0.000	0.000	0.030	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.034	0.000	0.000	0.000	0.000	0.000	0.106		0.000	0.152	
S497 23S	0.090	0.000	0.000	0.000	0.030	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.034	0.000	0.000	0.000	0.000	0.000	0.106	0.000		0.152	
S498 23S	0.186	0.152	0.152	0.152	0.148	0.152	0.152	0.154	0.152	0.152	0.152	0.152	0.150	0.152	0.156	0.152	0.152	0.152	0.123	0.152	0.152		

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Solazyme's *C. protothecoides* S106 strain was also compared to eight strains in Solazyme's collection that had previously (prior to 1992) been classified as *C. pyrenoidosa*. As *C. pyrenoidosa*, these strains had been the subject of several published nutritional studies. With the advent of modern molecular methods of identification, the species in the *Chlorella* genus have undergone significant taxonomic revision. In 1992, many *C. pyrenoidosa* strains were re-evaluated and reclassified as *C. sorokiniana* (Kessler and Huss, 1992) or *C. fusca* var. *vacuolata*. As indicated below, each test strain is identified by Solazyme designation, current classification and strain source; previous names and identical strains and/or other current or former names are also included as parenthetical information:

- (1) S129: *C. sorokiniana*, UTEX 1230 as *C. pyrenoidosa* (formerly *C. pyrenoidosa* (Starr & Zeikus 1987); *C. vulgaris* fo. *tertia* and *C. vulgaris* group; ATCC 22521; CCAP 211/8K as *C. vulgaris* f. *tertia*; CCAO 259 as *C. sorokiniana*; SAG 211-8K as *C. sorokiniana*; originally designated Tx 7-11-05);
- (2) S134: *C. sorokiniana* UTEX 1666, (formerly *C. pyrenoidosa*);
- (3) S135: *C. sorokiniana* UTEX 1670 (formerly *C. pyrenoidosa* (Starr & Zeikus 1987); originally designated B1E-B2P);
- (4) S140: *C. sorokiniana* UTEX B1810 (formerly *C. sorokiniana* var. *pacificensis* (Starr & Zeikus 1987));
- (5) S167: *C. fusca* var. *vacuolata* UTEX 251 (formerly *C. pyrenoidosa*; SAG 211-8B; CCAP 211/8B; CCAUP H 6401; ATCC 11469);
- (6) S168: *C. fusca* var. *vacuolata* UTEX 252 (formerly *C. pyrenoidosa*; aka *Scenedesmus* sp.; *Chlorella emersonii* var. *emersonii*; SAG 211-8C as *C. fusca*; CCAP 211/8C as *Chlorella emersonii* var. *globosa* (Shihira & Kraus 1965));
- (7) S545: *C. sorokiniana* UTEX 1664; and
- (8) S547: *C. sorokiniana* UTEX 1668 (formally *C. pyrenoidosa* (Starr and Zeikus 1987)).

Evaluation of the D values generated from partial plastidic 23S rDNA sequencing (Table 3) indicate that Solazyme strain S106, the *C. protothecoides* strain of interest, has D values ≥ 0.076 ($\leq 88\%$ homology), which shows slightly greater homology to five of the strains previously classified as *C. pyrenoidosa* than was seen between S106 and the 21 previously examined *C. vulgaris* isolates. In addition, Solazyme strains S129, S134 (UTEX 1666), S135 (UTEX 1670), S545 (UTEX 1664), and S547 (UTEX 1668) are seen to be identical (D value = 0.000 for all), which is not surprising, considering that UTEX strains 1664 through 1671 are mutants of UTEX 1663, a sub-isolate of Tx-7-11-05 (aka, UTEX 1230 or S129). As with the *C. vulgaris* isolates examined above, strain S106 is less closely related to most of the strains previously classified as *C. pyrenoidosa* than they are to one another. The exception is strain S168, whose 23S sequence shows less homology to the other strains in this set than S106 shows to the other strains.

In summary, partial plastidic 23S rDNA sequences were used to assess the genetic relatedness between S106, *C. protothecoides* and two genera of *Chlorella* previously studied and used as foods or dietary supplements, namely *C. vulgaris* and *C. pyrenoidosa*. Algal strain S106 is less closely related to these isolates than to S485 (type strain for *A. protothecoides*), against which S106 demonstrated 100% homology.

Table 3. Tabulated Distance values for 23S rDNA sequences of *C. protothecoides* S106 (Solazyme strain of interest) compared to other *Chlorella* strains S129, S134, S135, S140, S167, S168, S545, and S547. (Lower values indicate sequences that are more closely related, with '0.000' indicating identical sequences. Higher values indicate more divergent sequences.)

	S106 23S	S129 23S	S134 23S	S135 23S	S140 23S	S167 23S	S168 23S	S545 23S	S547 23S
S106 23S		0.076	0.076	0.076	0.086	0.090	0.182	0.076	0.076
S129 23S	0.076		0.000	0.000	0.022	0.049	0.158	0.000	0.000
S134 23S	0.076	0.000		0.000	0.022	0.049	0.158	0.000	0.000
S135 23S	0.076	0.000	0.000		0.022	0.049	0.158	0.000	0.000
S140 23S	0.086	0.022	0.022	0.022		0.041	0.164	0.022	0.022
S167 23S	0.090	0.049	0.049	0.049	0.041		0.142	0.049	0.049
S168 23S	0.182	0.158	0.158	0.158	0.164	0.142		0.158	0.158
S545 23S	0.076	0.000	0.000	0.000	0.022	0.049	0.158		0.000
S547 23S	0.076	0.000	0.000	0.000	0.022	0.049	0.158	0.000	

Common or Usual Name:

The common name of WAP has been defined as “Whole Algal Protein”. Synonyms include Algal Powder, Algal Protein, Algalin Protein, Whole Algalin Protein.

B. Regulatory status

WAP from the dried biomass of *C. protothecoides* S106 has not been approved for use in food by FDA,²⁴ FEMA,²⁵ USDA,²⁶ or EU. In Australia, however, consultation has indicated that WAP is not considered a Novel Food according to Australian Regulation (Lobeau, 2013). In the U.S., algalin oil from *C. protothecoides* S106, to be used as a food oil in a variety of foods excluding meat and poultry products, holds GRAS status and was notified to FDA as GRAS Notification 000384.²⁷ High Lipid Algalin Flour (HLAF), also from *C. protothecoides* S106 (cultured under nitrogen depleted conditions), to be used as a partial replacement for cream, milk, eggs/egg yolks, and/or butter/shortening in baked goods, beverages, dairy and egg products, sauces, gravies, margarines, salad dressings, and soups, holds GRAS status and was notified to FDA as GRAS Notification 000469.²⁸

C. Composition

The chemical composition of WAP is summarized in Table 4. WAP is a mixture of fiber, ash, protein, fat, and sucrose.

²⁴ FDA = United States Food and Drug Administration

²⁵ FEMA = Flavor & Extract Manufacturers Associations

²⁶ USDA = United States Department of Agriculture

²⁷ <<http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=384>>; site accessed March 31, 2014.

²⁸ <<http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=469>>; site accessed March 31, 2014.

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Table 4. Typical analysis of the major components of WAP

Analysis	Batch Analysis Results (n = 3)	
	Range	Average
Moisture (%)	4.8 – 8.0	6.1
Fiber (%)	10.3 – 20.3	16.3
Ash (%)	7.5 – 8.7	7.9
Protein (%)	50.0 – 56.4	52.4
Fat (%)	15.0 – 16.6	15.8
Sucrose	3.9 – 6.3	5.4

WAP = Whole Algal Protein

D. Method of Manufacture of WAP

WAP is manufactured by fermenting and harvesting cultures of *C. protothecoides* S106. A pure, clonally isolated culture is initially used to prepare a master seed bank from which working seed vials are prepared. Three samples from the master and each working seed bank are characterized by molecular genotyping to demonstrate that they are genetically identical (*i.e.*, 100% homology between the six chromosomal footprints and 100% homology between their 23S ribosomal deoxyribonucleic acid (DNA) sequences).²⁹ For a production lot, a cryo-preserved working seed vial is thawed and the contents used to inoculate a flask culture, which is transferred into larger flasks at mid-log phase, and then to standard, industrial seed fermenters. Throughout the aseptic fermentation process, pH, temperature, agitation and aeration rates are controlled, and glucose (carbon source) and nutrients are added. Protein production is induced during fermentation by ensuring that glucose is available as the carbon source and inorganic nitrogen is not restricted. Following completion of fermentation, the cells are washed to remove the medium and other non-biomass related materials and concentrated. The cells are inactivated by pasteurization and separated from the culture broth by centrifugation. After concentration, the pH is adjusted to neutral and food-grade antioxidants are added. The biomass, primarily whole cells, is then dried, optionally milled, and packaged. If needed, food grade flow agents may be added to assist processing through drying and packaging; at present the use of such agents is not part of the standard process. All ingredients used during manufacture are safe and suitable.³⁰ A graphical depiction of the manufacturing process is presented in Figure 2.

The final product, WAP, is available in quantities of 15 – 25 kg packed in a product bag (food-grade, poly-lined, 3-ply heat-sealed Kraft bag) and stored at temperatures < 25 °C, protected from direct sunlight.

²⁹ When tested, the six 23S ribosomal DNA sequences also demonstrated 100% identity to the 23S reference sequence for the original *C. protothecoides* S106 isolate.

³⁰ Regarding labeling concerns related to the eight major food allergens, two of the antioxidants that may be added to the finished product were derived from soy. All manufacturing ingredients are presented in the attached Dossier in Support of the Generally Recognized as Safe (GRAS) Status of WAP as a Food Ingredient.

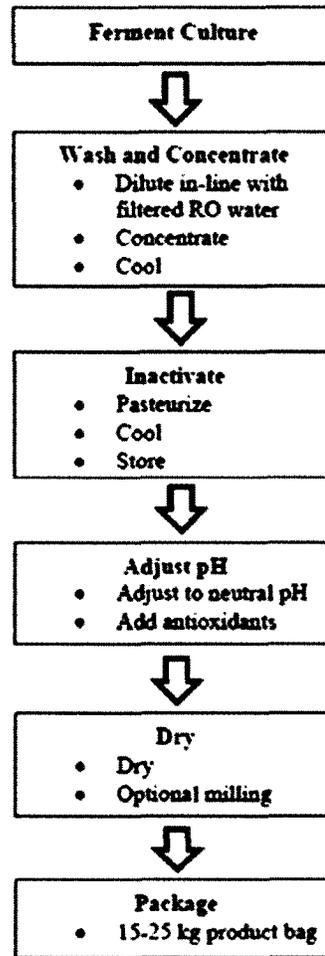


Figure 2. WAP production schematic

E. Specifications for Food Grade WAP

Specifications provided in Table 5 for bulk WAP include appearance, lead, arsenic, mercury, cadmium, chromium, cobalt, bacteria, yeast and mold, and the absence of *Escherichia coli*, *Salmonella* and *Pseudomonas aeruginosa*.

Table 5. Specifications for WAP

Analysis	Method	Specification	Batch Analysis Results (<i>n</i> = 3)	
			Range	Average
Appearance	C-M-00023-000 Appearance ^a	Pale yellow to green powder/flake	Conforms	Conforms
Moisture (%)	AOAC 930.15	≤ 10	4.8 – 8.0	6.1
Fiber (%)	AOAC 991.43	5 – 25	10.3 – 20.3	16.3
Ash (%)	AOAC 942.05	< 10	7.5 – 8.7	7.9
Protein (%)	AOAC 990.03	40 – 75	50.0 – 56.4	52.4
Fat (%)	AOAC 954.02	5 – 25	15.0 – 16.6	15.8
Sucrose (%)	AOAC 980.13	0 – 10	3.9 – 6.3	5.4
Heavy metals				
Lead (ppm)	EPA 3050/6020 USP 730	< 0.5	<0.01 – 0.039	0.026
Arsenic (ppm)	EPA 3050/6020 USP 730	< 0.2	0.031 – 0.24	0.101
Mercury (ppm)	EPA 3050/6020 USP 730	< 0.1	< 0.005	< 0.005
Cadmium (ppm)	EPA 3050/6020 USP 730	< 0.1	< 0.001 – 0.005	0.003
Chromium (ppm)	EPA 3050/6020 USP 730	< 2	0.044 – 0.367	0.174
Cobalt (ppm)	EPA 3050/6020 USP 730	< 0.1	<0.01 – 0.046	0.032
Microbiological Limits				
Aerobic Plate Count (cfu/g)	AOAC 990.12	< 5,000	< 10 – 2,700	907
Coliform (cfu/g)	AOAC 966.24	≤ 5	< 3	< 3
<i>E. coli</i> (in 10 g)	USP 32, NF 27, 2009	Negative	Negative	Negative
Staphylococci (in 10 g)	USP 32, NF 27, 2009	Negative	Negative	Negative
Salmonella (in 25 g)	AOAC 2004.03	Negative	Negative	Negative
<i>Pseudomonas aeruginosa</i> (in 10 g)	USP 32, NF 27, 2009	Negative	Negative	Negative
Yeast (cfu/g)	Chapter 18, FDA-BAM, 7 th ed.	< 100	< 10	<10
Mold (cfu/g)	Chapter 18, FDA-BAM, 7 th ed.	< 100	< 10 to 10	< 10

^a C-M-00023-000 Appearance (Solazyme, internal method)

AOAC = Association of Analytical Chemists; cfu = colony-forming units; EPA = Environmental Protection Agency; FDA-BAM = Food and Drug Administration Bacteriological Analytical Manual; g = gram; *n* = number; ppm = parts per million; USP = United States Pharmacopeia; methods available upon request; WAP = Whole Algal Protein

3. Self-Limiting Levels of Use

The quantity of WAP used as a dietary protein, analogous to soy- and animal-based proteins, would be self-limiting due to potential unpalatability.

4. Estimated Daily Intake

The intake profile (amount and frequency) by individuals in USDA's What We Eat in America (WWEIA) Continuing Survey of Food Intakes by Individuals 2003-2004 (Dwyer *et al.*, 2003)³¹ was used to calculate the estimated daily intake (EDI) of WAP for individuals consuming the food groups selected for the addition of WAP as described below, and in the attached Dossier in Support of the Generally Recognized as Safe (GRAS) Status of WAP as a Food Ingredient. The individual foods selected for addition of WAP are provided in Appendix 1 of this document. WAP will be added only to foods for which a standard of identity does not exist.

The means and 90th percentile EDIs were calculated only for WAP intake following addition of WAP to the selected food groups. The means and 90th percentile EDIs were not calculated for current WAP intake from natural sources as no information regarding current intakes of WAP from natural sources was discovered during a comprehensive search of the published literature. WAP added to the selected foods at the levels specified in Appendix 1 would provide a mean and 90th percentile WAP consumption of 2.32 and 5.56 g/day, respectively (Table 6).

Table 6. Predicted intake of WAP following supplementation of selected foods at the indicated levels (Appendix 1) for individuals consuming selected supplemented foods

WAP intake from:	Per User (mg/day)	
	Mean	90 th Percentile
Possible maximum consumption with WAP as an added ingredient to food	2,319	5,562

WAP = Whole Algal Protein

5. Absorption, Distribution, Metabolism and Elimination (ADME)

The proteins,³² lipids, and carbohydrates found in WAP (a whole *Chlorella protothecoides* S106 algal protein) are expected to be digested, absorbed, metabolized and excreted through the same normal physiological processes by which plant materials common to the human diet are digested.

6. Basis of GRAS Determination

The determination that WAP is GRAS is on the basis of scientific procedures, as described below and in the attached Dossier in Support of the Generally Recognized as Safe (GRAS) Status of WAP as a Food Ingredient. On the basis of the data and information described

³¹ USDA (2006) What We Eat In America, NHANES 2003-2004; Documentation and Data Files. U.S. Department of Agriculture, Agriculture Research Service; <http://www.ars.usda.gov/Services/docs.htm?docid=15044>; site visited October 17, 2013.

³² Protein bioavailability from the whole cell WAP product was demonstrated to be 87.4% in an unpublished study using a dynamic gastrointestinal (GI) model simulating the upper GI tract (TNO, 2012).

below and in the attached dossier and other publicly available information, there is consensus among experts qualified by scientific training and experience to evaluate the safety of substances added to food, that WAP is GRAS under the intended conditions of use.

6.1. Acute studies

No acute toxicity studies related to *C. protothecoides* or *A. protothecoides* were discovered in the scientific literature.

6.2. Short term repeated-dose studies

In a 28-day repeated-dose toxicity study (Day *et al.*, 2009) HSD:SD[®] rats ($n = 10/\text{sex}/\text{group}$) were each provided diets *ad libitum* containing 0 ppm (placebo control), 25,000 ppm (low-dose), 50,000 ppm (mid-dose) and 100,000 ppm (high-dose) ground yellow, high-lipid³³ *C. protothecoides* S106 biomass. The test diets for the Day *et al.* (2009) study were formulated using the AIN-93G Rodent Diet (Research Diets, Inc., New Brunswick, NJ) as the basal diet, to which sufficient test substance was added to achieve the target concentrations and to ensure comparable fat, protein and carbohydrate content across dose groups. The low-, mid-, and high dose diets were equivalent to 1794, 3667, and 7557 mg/kg bw³⁴/day, respectively, in males and 1867, 3918, and 8068 mg/kg bw/day, respectively, in females. Following the treatment period, the rats from each group were terminated on Day 31 (males) or Day 32 (females). This study was performed in compliance with OECD³⁵ Guidelines for the Testing of Chemicals, Section 4 (Part 407): Health Effects, *Repeated Dose 28-day Oral Toxicity Study in Rodents* (1995) and with Good Laboratory Practice (GLP) in accordance with OECD Principles of Good Laboratory Practice (as revised in 1997)³⁶ and US FDA GLP: 21 CFR 58, 1987.

The daily administration of the ground yellow, high-lipid *C. protothecoides* biomass material at dietary concentrations up to 100,000 ppm in the feed was well-tolerated by the rats. Consumption did not affect health or growth as measured by viability, appearance, behavior, body weight, body weight gain, food consumption, or food efficiency. No treatment-related effects were identified in the ophthalmology, urinalysis, hematology, clinical chemistry, gross pathology, organ weights, or histopathology of animals in any group. Although statistical significance was shown for several parameters, none were attributable to ingestion of the test substance because the changes were noted only sporadically, did not demonstrate a dose-response relationship, were within the ranges historically observed in the age and strain of rats used in this study, and/or were also observed in the control group. These observations are summarized below:

(a) One control female died from suspected accidental overdose of anesthesia on Day 29 during orbital sinus bleeding. This mortality was an isolated incident and not related to the test substance. (b) Incidental findings during clinical observation included a scab on the head of one mid-dose male (Days 2-6) and an abrasion on the nose of one low-dose female (Day 3). (c) A minor unilateral observation was also made in one control female during ophthalmologic examination on Day 25. This finding was confined to a single individual and not associated with

³³ 48% lipid; 6% protein

³⁴ bw = body weight

³⁵ OECD = Organisation for Economic Co-operation and Development

³⁶ OECD Environmental Health and Safety Publication, Series on Principles of Good Laboratory Practice and Compliance Monitoring – Number 1. Environment Directorate, Paris 1998.

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the test substance. (d) A minimally significant increase in food consumption was observed in high-dose males during Week 2 ($P < 0.05$) compared to the control group. Although food consumption by high-dose males did not differ significantly from the control group during the other three weeks, overall food consumption (Days 0-28) was also statistically higher for high-dose males compared to the controls ($P < 0.05$). Because increased consumption was slight and not accompanied by corresponding changes in body weight or food efficiency, the finding was not toxicologically significant and not necessarily related to the test substance. (e) A statistically significant decrease in mean food efficiency was reported in mid-dose males during Week 3 ($P < 0.05$) compared to control males. This decrease was short term and, therefore, judged to be incidental and not related to the test substance. (f) Statistically significant decreases in mean corpuscular hemoglobin concentration (MCHC) and absolute basophil concentrations were noted for the males of the high-dose and low-dose groups, respectively, compared to the control group ($P < 0.05$ for both). Because these Day 29 hematology findings were not accompanied by any other clinical or histopathologic change and no dose-dependent relationship was demonstrated, the findings were judged to be not attributable to treatment. (g) A significant increase in the absolute large unstained cell concentration in mid-dose group females compared to the control group ($P < 0.05$) was not found to be toxicologically relevant because the change was sporadic (not present at Day 29) and did not demonstrate a dose-dependent relationship. (h) Clinical chemistry results revealed statistically significant increases in creatinine in mid-dose males and triglycerides in high-dose males on Day 15 and blood urea nitrogen in mid-dose males on Day 29 compared to the control group ($P < 0.05$ for all). Cholesterol in high-dose females was significantly elevated compared to the control group ($P < 0.05$) on Day 15. Because these findings did not present with consistency, dose-response relationship, or corresponding clinical or histopathological changes, they were not related to the test substance. (i) Clinical chemistry results from Days 15 and 29 revealed statistically significant increases in alkaline phosphatase in high-dose group males ($P < 0.05$ for both). These findings did not demonstrate a dose-response relationship and were not present in females, nor were they accompanied by corresponding clinical or histopathologic change, nor were there corresponding changes in liver or kidney weight; they were, therefore, judged not to be toxicologically relevant. (j) Findings for Day 29 urinalysis included a statistically significant increase in specific gravity and a decrease in urine volume in mid-dose females compared to controls ($P < 0.05$ for both). These changes were not dose-related nor were they accompanied by clinical or histopathologic change, nor were liver or kidney weights affected. The changes were judged not to be treatment related. (k) Macroscopic observation revealed a statistically significant increase in absolute adrenals ($P < 0.05$), adrenals-to-body ($P < 0.01$) and adrenals-to-brain ($P < 0.05$) weights in the mid-dose males compared to the control group values. Due to the absence of clinical or histopathological changes, these observations were incidental.

Under the conditions of this study, the no-observed-adverse-effect level (NOAEL) for ground yellow, high-lipid *C. protothecoides* biomass in the diet was 100,000 ppm, the highest dietary concentration provided in the study, which corresponded to a dietary NOAEL of 7557 mg/kg bw/day in male rats and 8068 mg/kg bw/day in female rats.

The findings of an unpublished 28-day trial of dietary *C. protothecoides* (strain not specified) in the Syrian golden hamster (sex not specified; $n = 15$ /group) (Harding and Jones, 2008) corroborated the findings of the published Day *et al.* (2009) study. Control animals received a hyperglycemic-hypercholesterolemic diet *ad libitum*; the treatment groups received

the same diet supplemented with 2.5% or 5.0% (w/w)³⁷ *C. protothecoides* (equivalent to ~3000 or ~6000 mg/kg bw/day, respectively). No statistically significant effect was reported in body weight, food intake, body composition (e.g., percentage body fat), or plasma triglycerides, cholesterol, protein, or albumin for the treatment groups compared to the control group. Compared to the control group, plasma glucose was significantly reduced in both low- and high-dose groups ($P < 0.05$ for both), but without affecting triglycerides or total cholesterol. Although plasma insulin concentrations for the treatment group did not differ significantly compared to the control group, plasma insulin in the 2.5% group was significantly elevated compared to plasma insulin in the 5.0% group ($P < 0.05$). Oxygen consumption was also found to be significantly greater in the 2.5% group than in the control group ($P < 0.05$), but without affecting production of carbon dioxide. The authors concluded that *C. protothecoides* in a hyperglycemic-hypercholesterolemic diet decreased plasma glucose independent of plasma insulin and further determined that “consumption of *C. protothecoides* at 2.5% and 5% of total diet (w/w) [~3000 and ~6000 mg/kg bw/day] appeared to be safe as there was no difference between groups in the liver production total plasma protein or albumin” (Harding and Jones, 2008).

In summary, although statistically significant effects were noted for several endpoints in a 28-day repeated-dose study in rats (Day et al., 2009), these were not found to be related to administration of the test substance, a ground yellow, high-lipid³³ *C. protothecoides* S106 biomass material, or were not toxicologically relevant. Under the conditions of the study, the NOAEL for high-lipid *C. protothecoides* biomass in the diet was 100,000 ppm, the highest dietary concentration provided in the study, which corresponds to a dietary NOAEL of 7557 mg/kg bw/day in male rats and 8068 mg/kg bw/day in female rats. In a corroborative, unpublished 28-day repeated-dose study in hamsters, consumption of *C. protothecoides* up to ~6000 mg/kg bw/day was determined by the authors to be safe (Harding and Jones, 2008). The short-term repeated-dose toxicity studies in rodents are summarized in Table 7.

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³⁷ w/w = weight/weight
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Table 7. 28-Day short term repeated-dose toxicity studies in rodents

Duration	Species (#/dose group)	Dose/Route	Results/Notes	Reference
28 days	Rat (10M/10F)	0, 25,000, 50,000 and 100,000 ppm high-lipid <i>C. protothecoides</i> S106 in diet	Consumption of high-lipid <i>C. protothecoides</i> biomass was well-tolerated; NOAEL was 7557 mg/kg bw/day in males and 8068 mg/kg bw/day in females. No reported adverse effects.	Day <i>et al.</i> (2009)
28 days	Syrian golden hamster (15/group; sex not specified)	0, 2.5% and 5.0% <i>C. protothecoides</i> in diet (equivalent to 0, ~3000, and ~6000 mg/kg bw/d)	Plasma glucose significantly reduced in low- and high-dose groups compared to the control ($P < 0.05$ for both). Plasma insulin for treatment groups did not differ significantly compared to control, but plasma insulin in low-dose group was significantly elevated compared to high-dose group ($P < 0.05$). Oxygen consumption significantly greater in low-dose group than in control group ($P < 0.05$). Consumption of <i>C. protothecoides</i> at up to ~6000 mg/kg bw/day was well-tolerated.	Harding and Jones (2008); unpublished report; Corroborative

C. Protothecoides = *Chlorella protothecoides*; F = Female; M = Male; NOAEL = No Adverse Effect Level; ppm = parts per million; # = number; bw = body weight

6.3. Subchronic studies

6.3.1. WAP subchronic repeated-dose toxicity study

In a 13-week repeated-dose subchronic toxicity study (Szabo *et al.*, 2013), HSD:SD® rats ($n = 10/\text{sex}/\text{group}$) were provided *ad libitum* diets containing 0 ppm (Group 1 placebo control), 25,000 ppm (Group 2), 50,000 ppm (Group 3) and 100,000 ppm (Group 4) WAP (equivalent to 0, 1177, 2416, and 4805 mg/kg bw/day, respectively in males and 0, 1444, 2700, and 5518 mg/kg bw/day, respectively, in females), a pale yellow to green high protein powder composed of the dried milled biomass of *C. protothecoides* S106.³⁸ The test diets were formulated using the DIO Rodent® basal diet to which sufficient WAP was added to achieve the target concentrations and to ensure comparable fat, protein and carbohydrate content across dose groups. Following the treatment period, the rats from each group were terminated on Day 92 (males) or Day 93 (females). The study was performed in compliance with OECD Guidelines for Testing of Chemicals and Food Ingredients, Section 4 (Part 408): Health Effects, *Repeated Dose 90-Day Oral Toxicity Study in Rodents* (1998) and GLP in accordance with OECD Principles of Good Laboratory Practice (as revised in 1997)³⁶ and US FDA GLP: 21 CFR 58, 1987. The subchronic repeated-dose toxicity study for WAP in rodents is summarized in Table 8.

Daily exposure to WAP at dietary concentrations up to 100,000 ppm was well-tolerated by the rats. No test substance-related mortalities occurred during the study period. Consumption of WAP was not found to affect health or growth as measured by viability, condition, behavior, body weight, body weight gain, food consumption, or food efficiency. No treatment related effects were identified in the ophthalmology, urinalysis, hematology, clinical chemistry, gross pathology, absolute or relative organ weights, or histopathology of animals in any group.

³⁸ The test material used in this study (assayed to contain 59.9% protein) differs from the product material in that the test material was milled (> 30% cells disrupted); the product material is whole cell.

Although statistical significance was shown for several parameters, none were attributable to ingestion of WAP because the changes were incidental or noted only sporadically, were not accompanied by corresponding clinical or histopathologic changes, did not demonstrate a dose-dependent relationship, and/or were also observed in the control group. These observations are summarized below:

(a) One Group 1 (control) male died on Day 27 after receiving anesthesia for a teeth clipping procedure to address maloccluded upper incisors. No findings, including macroscopic or microscopic pathology, were discovered that explained the early mortality; this event was incidental and not related to the test substance. (b) Mean daily body weight gains for Group 4 males on Days 77-84 ($P < 0.01$), Group 3 females on Days 14-21 ($P < 0.01$), and Group 4 females on Days 49-56 ($P < 0.05$) were significantly increased compared to their corresponding control groups. These findings were incidental and not attributable to test substance exposure. (c) A statistically significant decrease in mean daily food consumption compared to the control group was observed in Group 2 males on Days 7-14 ($P < 0.05$), 21-35 ($P < 0.01$), 35-42 ($P < 0.05$), 42-49 ($P < 0.01$), 56-63 ($P < 0.05$), 63-77 ($P < 0.001$) and overall, Days 0-91 ($P < 0.01$); in Group 3 males on Days 63-77 ($P < 0.05$); and in Group 4 males on Days 7-14 ($P < 0.05$), 21-35 ($P < 0.05$), 49-56 ($P < 0.05$), 56-63 ($P < 0.01$), 63-70 ($P < 0.05$), 70-77 ($P < 0.01$), and overall, Days 0-91 ($P < 0.05$). In comparison, mean food efficiency significantly increased only in Group 4 males on Days 77-84 ($P < 0.01$); food efficiency in all other male treatment groups was comparable to the control group. Because decreased consumption was not dose-dependent, was minimal on an individual basis, and was not accompanied by decreases in body weight or food efficiency, the findings were not adverse. (d) Among females, mean daily food consumption significantly decreased only in Groups 3 and 4 and only on Days 28-35 ($P < 0.05$ and $P < 0.01$, respectively) while mean food efficiency increased significantly in Group 3 on Days 14-21 ($P < 0.01$) and Group 4 on Days 49-56 ($P < 0.05$), compared to the control group. Statistically significant changes in mean food consumption and efficiency observed among the female groups were sporadic and not dose-related and were therefore determined to be unrelated to treatment. (e) Statistically significant changes in urinalysis parameters on Day 91 were limited to decreased urine volume and pH in Group 2 males ($P < 0.05$ for both), compared to controls. These changes were not considered to be toxicologically relevant because they were not associated with any corresponding clinical or histopathologic change. (f) Hematology results for Day 91 revealed a statistically significant decrease in the absolute lymphocyte concentrations in Group 2 males compared to the control group ($P < 0.05$). Because this finding did not demonstrate a dose-dependent relationship and was not accompanied by corresponding clinical or histopathologic changes, it was not toxicologically relevant. (g) Clinical chemistry results from Day 91 revealed a statistically significant decrease in total cholesterol in Group 4 males, compared to control males ($P < 0.05$). This finding did not demonstrate a dose-dependent relationship,³⁹ nor was it accompanied by corresponding clinical or histopathologic changes; it was determined, therefore, not to be toxicologically relevant. (h) A soft, tan vascularized mass (12 x 6 x 9 mm) in the right epididymis in a Group 4 male on Day 92 corresponded histologically to a unilateral moderately sized sperm granuloma; the mass was of spontaneous origin. (i) A firm, tan, cervical subcutaneous, lobulated mass (10 x 10 x 5 mm) in a Group 1

³⁹ Although total cholesterol values in Group 2 and Group 3 males were suggestive of a dose-response trend, the values for these groups did not vary significantly from Group 1 control values; in addition, the individual values for these three groups largely overlapped.

female histologically identified as a mammary gland adenocarcinoma; an extrahepatic nodule (10 x 7 x 8 mm) on the dorsal aspect of the liver in another Group 1 female found to be composed of liver of normal morphology; and a lobulated diaphragmatic hernia in the liver in a Group 3 female histologically identified as a hepatodiaphragmatic nodule were each spontaneous and unrelated to the test substance.

In summary, although statistically significant effects were noted for several endpoints, none were attributable to ingestion of WAP because the changes were incidental or noted only sporadically, were not accompanied by corresponding clinical or histopathological changes, did not demonstrate a dose-dependent relationship, and/or were also observed in the control group. Under the conditions of this study, the NOAEL for WAP in the diet was 100,000 ppm, the highest dietary concentration provided in the study, which corresponds to a dietary NOAEL of 4805 mg/kg bw/day in male rats and 5518 mg/kg bw/day in female rats.

6.3.2. HLAf subchronic repeated-dose toxicity study

In a 13-week repeated-dose subchronic toxicity study (Szabo *et al*, 2012; FDA, 2013), HSD:SD[®] rats ($n = 10/\text{sex}/\text{group}$) were provided *ad libitum* diets containing 0 ppm (Group 1 placebo control), 25,000 ppm (Group 2), 50,000 ppm (Group 3) and 100,000 ppm (Group 4) HLAf (equivalent to 0, 1249, 2478, and 4807 mg/kg bw/day, respectively in males and 0, 1413, 2739, and 5366 mg/kg bw/day, respectively, in females). The test diets were formulated using the DIO Rodent[®] basal diet to which sufficient HLAf (a golden yellow high-lipid powder composed of the milled dried biomass of *C. protothecoides* S106) was added to achieve the target concentrations and to ensure comparable fat, protein and carbohydrate content across dose groups. Following the treatment period, the rats from each group were terminated on Day 93 (males) or Day 94 (females). This study was performed in compliance with OECD Guidelines and under GLP as described previously in Section 6.3.1. The subchronic repeated-dose toxicity study in rodents is summarized in Table 8.

Daily consumption of HLAf at dietary concentrations up to 100,000 ppm was well-tolerated by the rats in the 13-week study and did not affect health or growth as measured by viability, condition, behavior, body weight, body weight gain, food consumption, or food efficiency. No treatment-related effects were identified in the ophthalmology, urinalysis, hematology, clinical chemistry, gross pathology, organ weights, or histopathology of animals in any group. Although statistical significance was shown for several parameters, none were attributable to ingestion of HLAf because the changes were within the ranges historically observed in the age and strain of rats used in this study, did not demonstrate a dose-dependent relationship, lacked any clinical or histopathologic correlation, were noted only sporadically, and/or were also observed in the control group. These observations are summarized below:

(a) A firm, round, subcutaneous lesion on the left side of the neck (cranial) area in one Group 2 female was identified during pathological evaluation as an adenocarcinoma of mammary gland origin. The lesion was an isolated finding limited to this one female. (b) A statistically significant decrease in food consumption was observed in Group 4 males on Days 14-21, 35-56, and 84-91 ($P < 0.05$ for all) and overall, Days 0-91 ($P < 0.05$) as compared to the control group. Because decreased consumption occurred during three discrete weeks out of 13 weeks, was minimal on an individual basis regarding the Day 0-91 assessment and was not accompanied by decreases in body weight or food efficiency, the finding was not adverse. (c) A statistically significant increase in mean food efficiency in Group 3 females on Days 21-28 ($P <$

0.05) and overall 0-91 Days ($P < 0.01$), compared to control females was not dose-related and was therefore considered not to be treatment-related. (d) A statistically significant decrease in mean daily food consumption was reported for Group 4 females compared to the control group ($P < 0.01$) on Days 63-70. This decrease was short term and, therefore, was incidental and not related to the test substance. (e) A vitreal hemorrhage obscuring the optic nerve was reported in the right eye of a Group 1 (control) female on Day 90. The finding was interpreted to result from a sporadically occurring retinal vascular abnormality or hyaloid artery remnant. (f) Statistically significant increases in platelet concentration were noted for the females of Group 2 and Group 4 compared to the control group ($P < 0.05$ for all). Because these Day 91 hematology findings were not accompanied by any other clinical or histopathologic change and no dose-response relationship was demonstrated, the findings were not attributable to HLAf treatment. (g) A significant increase in the absolute reticulocytes in Group 3 females compared to the control group ($P < 0.05$) was not found to be toxicologically relevant because the change was sporadic, was not dose-dependent and not accompanied by any other clinical or histopathologic change. (i) Clinical chemistry results from Day 91 revealed statistically significant increases in alkaline phosphatase in Group 2 and Group 3 males, decreased aspartate aminotransferase in Group 2 females, and an increase in total protein in Group 2 females compared to their respective control groups ($P < 0.05$ for all). These findings did not demonstrate a dose-response relationship, nor were they accompanied by corresponding clinical or histopathologic change; they were, therefore, not toxicologically relevant. (j) A firm, white lesion in the right kidney of a Group 4 male corresponded to a neoplasm having morphologic features consistent with a renal mesenchymal tumor. The tumor was an isolated finding, limited to this one male. (k) The enlarged, pale, mottled thymus noted in a Group 2 male lacked any histologic correlate and was spontaneous in origin. (l) In comparison to controls, Group 3 male relative adrenal-, brain-, and testis-to-body weight ratios were significantly decreased, while the kidney-to-brain weight ratio was significantly increased ($P < 0.05$ for all). In Group 2 and 3 females, absolute liver weights, liver-to-body weight and liver-to-brain weight ratios were significantly increased when compared to the control group ($P < 0.01$ for all Group 2 parameters; $P < 0.05$ for Group 3). All changes were incidental and not toxicologically relevant as no change demonstrated a dose-dependent relationship and none was found in the high-dose group.

In summary, although statistically significant effects were noted for several endpoints, none were attributable to ingestion of HLAf because the changes were within the ranges historically observed in the age and strain of rats used in the study, did not demonstrate a dose-dependent relationship, lacked any clinical or histopathologic correlation, were noted only sporadically, and/or were also observed in the control group. Under the conditions of this study, the NOAEL for HLAf in the diet was 100,000 ppm, the highest dietary concentration provided in the study, which corresponds to a dietary NOAEL of 4807 mg/kg bw/day in male rats and 5366 mg/kg bw/day in female rats.

Table 8. 90-Day subchronic repeated-dose toxicity study in rodents

Duration	Species (#/dose group)	Dose/Route	Results/Notes	Reference
13 weeks	Rat (10M/10F)	0, 25,000, 50,000 and 100,000 ppm WAP in diet (equivalent to 0, 1177, 2416, and 4805 mg/kg bw/day, respectively in males and 0, 1444, 2700, and 5518 mg/kg bw/day, respectively, in females)	Dietary WAP was well-tolerated; NOAEL was 4805 mg/kg bw/day in males and 5518 mg/kg bw/day in females; No adverse effects were reported	Szabo <i>et al.</i> (2013).
13 weeks	Rat (10M/10F)	0, 25,000, 50,000 and 100,000 ppm HLAFF in diet (equivalent to 0, 1249, 2478, and 4807 mg/kg bw/day, respectively in males and 0, 1413, 2739, and 5366 mg/kg bw/day, respectively, in females)	Dietary HLAFF was well-tolerated; NOAEL was 4807 mg/kg bw/day in males and 5366 mg/kg bw/day in females; No adverse effects	Szabo <i>et al.</i> (2012); FDA (2013).

F = Female; HLAFF = High Lipid Algal Flour; M = Male; NOAEL = No Adverse Effect Level; ppm = parts per million; # = number; WAP = Whole Algal Protein

6.4. Other studies

The roles that three different algal strains (*C. protothecoides* 902,⁴⁰ *P. zopfii* 822,⁴¹ and *C. vulgaris* 1206⁴²) could play in the detoxification of chlordecone-poisoned rats were explored and compared by Pore *et al.* (1984) in a series of inter-related studies. In previous work (Conte and Pore, 1973; Pore, 1984), both the living cells and cell walls of *C. protothecoides* 902 had been shown to have a high binding affinity for chlordecone, similar to that of cholestyramine.⁴³ All animals in the current study were female SD rats (175-199 g; 43-51 days of age) and all received 2.8-5.2 μ Ci of ¹⁴C-chlordecone⁴⁴ plus ~0.05 mg unlabeled chlordecone in corn oil *via* intraperitoneal injection on Day 0. For the initial ingestion study, a subset of the rats (*n* = 6) received a diet supplemented with 4 g/day (20-23 g/kg bw/day) of freeze-dried *C. protothecoides* 902 starting on Day 4 and continuing through Day 17; the corresponding control group (*n* = 6) received the control diet without algal supplementation. As determined by scintillation counting of feces (the primary means of chlordecone excretion) collected on Days 4-13, the half-life (*t*_{1/2}) of chlordecone in the rat was 19.0 days for the *C. protothecoides*-treated group and 35.5 days for the control animals. All rats in the initial study were sacrificed on Day 17. In confirmation of the feces-based findings, the residual ¹⁴C-chlordecone radioactivity of abdominal adipose samples⁴⁵

⁴⁰ 902 *Chlorella protothecoides* BTR (Biotech Research, Inc.) 902; reported depository entries includes ATCC 75667.

⁴¹ *Prototheca zopfii* Krüger (Cooke 1962/62-344); aka *P. stagnora* 62-344, and *P. moriformis*; depository designations include ATCC 16527 and UTEX 1442; ATCC classifies the microorganism as BSL 1.

⁴² No additional strain information is available.

⁴³ Cholestyramine is an ion exchange resin and the primary material used to treat cases of chlordecone intoxication. Cholestyramine binds to chlordecone in the intestine (during primary poisoning or later when absorbed chlordecone is secreted back into the intestine) which prevents reabsorption and allows for elimination with the feces. Without assistance, chlordecone is neither detoxified by the body nor is it effectively eliminated.

⁴⁴ 10 mCi/mmol ¹⁴C-chlordecone

⁴⁵ Retro-kidney and mesentary

from *C. protothecoides*-treated rats was significantly lower than radioactivity in the control group ($P < 0.01$) (Pore, 1984).

In a series of shorter four-day studies (Pore *et al.*, 1984), the $t_{1/2}$ of chlordecone in the rat (as determined by radioactivity in the feces) was 18.7 days for the *C. protothecoides*-treated group ($n = 6$), significantly less than the 40.3 days for the control group ($n = 6$) ($P < 0.01$). When *C. protothecoides* cell walls (isolated by hydrolysis in sulfuric acid from 4/g/day equivalent of *C. protothecoides* 902) were fed to chlordecone-treated rats ($n = 6$), the $t_{1/2}$ was 19.4 days, comparable to the freeze-dried intact cells. In comparison, neither administration (4 g/day) of intact *Prototheca zopfii* nor of intact *C. vulgaris* exhibited a similar effect on the chlordecone $t_{1/2}$ in the rat; measured half-lives (based on radioactivity of feces) were 32.7 days for *C. vulgaris* and 29.5 days for *P. zopfii*. The authors proposed the difference in chlordecone $t_{1/2}$ was due to the presence of relatively large amounts of sporopollenin⁴⁶ (2% of cell dry weight) in the outer trilaminar layer of *C. protothecoides* cell walls. No sporopollenin is present in the cell walls of *C. vulgaris*; intermediate amounts are present in *P. zopfii* cell walls. The authors did not report any adverse effects in the rats that were associated with consumption of living *C. protothecoides* 902 cells or isolated cell walls.

In summary, exposure of chlordecone-treated rats to either living cells or the cell walls of *C. protothecoides* 902 (20-23 g/kg bw/day) resulted in a significantly decreased chlordecone $t_{1/2}$ compared to the control group. An intermediate effect was observed when intact *P. zopfii* was consumed, but no effect occurred when rats ate intact *C. vulgaris*. The authors believed the reduction of ¹⁴C-chlordecone was due to the high amounts of sporopollenin (~2% dry cell weight) in the *C. protothecoides* cell walls. *C. vulgaris* cell walls contained no sporopollenin and *P. zopfii* contained an intermediate amount. The authors did not report any adverse effects associated with consumption of the living cells or the cell walls of the algae. This study is summarized in Table 9.

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⁴⁶ Relatively rare but natural oxidative carotenoid polymers that occur in a few microorganisms, such as *C. protothecoides* and *Prototheca* spp. (but not *C. vulgaris*) and some plants (Pore, 1984); the more common plant sporopollenin biopolymers (composed primarily of long saturated aliphatic chains), such as those found in the outer (exine) walls of pollens and spores were noted as not being active in the isolation of chlordecone (Pore, 1984).

Table 9. Other studies in the rodent model

Method	Concentration	Subjects (#/group)	Results	Reference
Detoxification study, 17-d	0 or 4 g/d (0 or 20-23 g/kg bw/d) live <i>C. protothecoides</i> 902 in feed after rats received 2.8-5.2 μ Ci of 14 C chlordecone plus ~0.05 mg unlabeled chlordecone in corn oil <i>via i.p.</i> injection (Day 0)	Female SD rats (6/group)	Chlordecone $t_{1/2}$ in rats fed live 902 was significantly reduced (18.7-19.0 d), compared to the control group (35.5 d) ($P < 0.01$)	Pore <i>et al.</i> (1984)
Detoxification studies, 4-d	Live <i>P. zopfii</i> 822, live <i>C. vulgaris</i> 1206 or cell walls of <i>C. protothecoides</i> 902 in feed after rats received 2.8-5.2 μ Ci of 14 C chlordecone plus ~0.05 mg unlabeled chlordecone in corn oil <i>via i.p.</i> injection (Day 0)	Female SD rats (6/group)	Chlordecone $t_{1/2}$ in rats fed 902 cell walls (19.4 d) was comparable to $t_{1/2}$ in live-902 group; Chlordecone $t_{1/2}$ in rats fed live 822 or live 1206 were 29.5 and 32.7 d, respectively, and did not differ from the control. The authors did not report any adverse effects to consumption of live cells or cell walls.	Pore <i>et al.</i> (1984)

d = day; *i.p.* = Intraperitoneal; $t_{1/2}$ = half life

6.5. Genotoxicity

6.5.1. Mutagenicity assay for WAP

The mutagenic potential of WAP³⁸ was evaluated by bacterial reverse mutation assay (*i.e.* Ames test) using standard plate incorporation (Experiment I) and pre-incubation (Experiment II) methods with and without S9 metabolic activation⁴⁷ (Szabo *et al.*, 2013). Tester strains included *Salmonella typhimurium* TA100 and TA1535 and *Escherichia coli* WP2uvrA for detection of base-pair substitutions, and *S. typhimurium* TA98 and TA1537 for detection of frame-shift mutations. The assays were performed in compliance with international guidelines⁴⁸ and conducted under GLP conditions.⁴⁹ In the pre-experiments using test strains TA 98 and TA 100, 5000 μ g/plate WAP was selected as the highest dose for all test strains and conditions due to a lack of cytotoxicity. In Experiment I, six dose levels (31.6, 100, 316, 1000, 2500 and 5000 μ g/plate) were prepared and tested; in Experiment II, seven dose levels (3.16, 10.0, 31.6, 100, 316 and 1000 μ g/plate) were prepared. In the absence of activation, sodium azide (NaN₃) served as positive control for *S. typhimurium* TA100 and TA1535, 4-nitro-*o*-phenylene-diamine (4-NOPD) for *S. typhimurium* TA98 and TA1537 and methylmethanesulfonate (MMS) for *E. coli*

⁴⁷ S9 metabolic activation = The supernatant fraction of rat liver homogenate (derived from male Wistar and SD rats pretreated with the inducers phenobarbital and β -naphthoflavone) mixed with Cofactor-1.

⁴⁸ Ninth Addendum to OECD Guidelines for Testing of Chemicals, Section 4, No. 471, "Bacterial Reverse Mutation Test", adopted July 21, 1997 and Commission Regulation (EC) No. 440/2008 B.13/14, "Mutagenicity – Reverse Mutation Test using Bacteria", dated May 30, 2008.

⁴⁹ Chemikaliengesetz ("Chemicals Act") of the Federal Republic of Germany, Appendix 1 to § 19a as amended and promulgated on June 20, 2002 (BGBl. I Nr. 40 S. 2090), revised October 31, 2006 (BGBl. I Nr. 50 S. 2407); and OECD Principles of Good Laboratory Practice (as revised in 1997), in the OECD Environmental Health and Safety Publications; Series on Principles of Good Laboratory Practice and Compliance Monitoring - Number 1.

Environment Directorate, Organisation for Economic Cooperation and Development, Paris 1998

WP2uvrA. In the presence of activation, the positive control for all bacterial strains was 2-aminoanthracene (2-AA). The negative and solvent control for all tests was distilled water.

WAP was observed to precipitate at concentrations of 1000 µg/plate and higher, in all test strains, in the presence and absence of S9 mix in Experiments I and II. Cytotoxic effects of the test substance were observed in test strain TA 1537 at the two highest concentrations (2500 and 5000 µg/plate) in Experiment I with metabolic activation. Cytotoxic effects of the test substance were observed at 2500 µg/plate, the highest concentration tested, in Experiment II without metabolic activation. Although a reduction in the number of revertants in test strain TA 1537 at a concentration of 316 µg/plate in Experiment II (without activation) met the criteria for cytotoxicity, no dose-response relationship was in evidence and the effect was determined not to be biologically relevant. Regardless of the presence or absence of metabolic activation, dose-dependent increases in the number of revertant colonies of greater than twice the negative control values were not observed in any strain treated with WAP in either experiment. Under the conditions of this study, WAP did not cause gene mutations by base pair changes or frame shifts in the genomes of the tester strains and was, therefore, not a mutagen.

6.5.2. Mutagenicity assay for HLAf

The mutagenic potential of the closely related HLAf material was also evaluated by bacterial reverse mutation assay (Szabo *et al*, 2012; FDA, 2013) using standard plate incorporation (Experiment I) and pre-incubation (Experiment II) methods with and without S9 metabolic activation⁵⁰ under the same conditions^{49,48} as those previously described for the testing of WAP. Based on pre-experiments, 5000 µg/plate HLAf was selected as the highest dose for all test strains and conditions, except for TA 1537 in Experiment II without activation, where 1000 µg/plate was selected as the highest dose due to cytotoxic effects of the test substance. For the 5000 µg/plate series, five lower dose levels (31.6, 100, 316, 1000 and 2500 µg/plate) were also prepared and tested; for the 1000 µg/plate series, six lower dose levels (1.00, 3.16, 10.0, 31.6, 100 and 316 µg/plate) were also prepared. In the absence of activation, NaN₃ served as positive control for *S. typhimurium* TA100 and TA1535, 4-NOPD for *S. typhimurium* TA98 and TA1537 and MMS for *E. coli* WP2uvrA. In the presence of activation, the positive control for all bacterial strains was 2-AA. The negative control for all tests was distilled water and the solvent control was dimethyl sulfoxide (DMSO).

Precipitation of HLAf was visually observed in all test strains at concentrations of 2500 µg/plate and higher in Experiment I and II, in the absence of S9 mix, and in all test strains at a concentration of 5000 µg/plate in Experiment I and II in the presence of S9. In Experiment I cytotoxic effects of the test substance were observed in test strain TA 1537 at concentrations of 316 µg/plate and higher, without metabolic activation, and at concentrations of 2500 µg/plate with metabolic activation. In Experiment II these same effects were observed in test strains TA 98 and TA 100 at concentrations of 316 µg/plate and higher, without metabolic activation, and in test strain TA 1537 at concentrations of 10.0 µg/plate and higher, without metabolic activation, and concentrations of 316 µg/plate and higher, with metabolic activation. Regardless of the presence or absence of metabolic activation, dose-dependent increases in the number of revertant colonies of greater than twice the negative control values were not observed in any strain treated with HLAf in either experiment. Under the conditions of this study, HLAf was not a mutagen.

⁵⁰ Similar to the S9 mix used to test WAP, except that the supernatant fraction of rat liver homogenate was derived only from male Wistar rats.

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6.5.3. Clastogenicity assay for WAP

The clastogenic potential of WAP³⁸ was evaluated using the *in vivo* bone marrow chromosome aberration assay in the mouse (Szabo *et al.*, 2013). The assays were performed under GLP conditions⁴⁹ and in compliance with international guidelines.⁵¹ Prior to the main experiment, a range-finding study (OECD Guideline 475) was used to determine the maximum tolerated dose (MTD) of WAP. Due to a lack of observed toxicity, the MTD defaulted to 2000 mg/kg bw, the highest dose evaluated for the assay (in accordance with OECD Guideline 475). This dose was then selected as the maximum in the main study. Prepared in physiological saline one hour before treatment, WAP at a dose of 2000 mg/kg bw was administered *via* oral gavage to ten male and ten female NMRI mice (7 – 13 weeks old) in a single application at study initiation. Negative control animals ($n = 10/\text{sex}$) were administered the vehicle in similar volumes (10 ml/kg bw). Positive control mice ($n = 5/\text{sex}$) received 40 mg/kg bw cyclophosphamide (CPA) in physiological saline *via* i.p. injection. Bone marrow cells were harvested 24 and 48 h later. Exposure times were 24 hours and 48 hours post-administration for the treatment and negative control groups ($n = 5/\text{sex}/\text{group}/\text{time}$) and 24 hours post-administration for the positive control group. After bone marrow cells were fixed and stained, 100 metaphases⁵² were scored for cytogenic damage to determine the incidence of structural chromosomal aberrations (*i.e.*, breaks, fragments, deletion exchanges, chromosomal disintegrations and gaps). In addition, a minimum of 1000 cells were evaluated for cytotoxicity to determine the mitotic index (*percentage* of cells in mitosis).

The mean values of aberrant cells in the 24- and 48-hour negative control and test groups (Table 10) remained within the range of the historic negative controls (0 – 5% in male and 0 – 3% in female mice from years 2001–2010). No dose-dependent, biologically relevant or statistically significant increase in the incidence of aberrant cells was observed in any 24-hour or 48-hour dose group compared to its corresponding negative control group. The mitotic index values for the 24-hour male and female test groups and 48-hour female test group remained in the range of the corresponding negative control (Table 10); no statistically significant change to the mean mitotic index occurred in these groups. For the 48-hour male test group, however, the mean mitotic index value was significantly lower than the value for the corresponding negative control ($P < 0.01$) (Table 10). Because the decrease was determined to be an effect of biological variability among the animals, it was not biologically relevant. The validity of the chromosome aberration assay was verified by (1) the weight variation of the mice not exceeding $\pm 20\%$ of the pre-dose mean weight of each sex, (2) the lack of biologically significant increases in aberrant cell values in the negative control group, and (3) the biologically significant induction of aberrant cells in the positive control group ($P < 0.01$). Under the conditions of this study, WAP did not induce cytotoxicity or structural chromosome aberrations and was, therefore, not clastogenic.

⁵¹ The Ninth Addendum to OECD Guidelines for Testing of Chemicals, Section 4, No. 475, “Mammalian Bone Marrow Chromosome Aberration Test”, adopted July 21, 1997 and Commission Regulation (EC) No. 440/2008 B.11, “Mutagenicity – Mammalian Bone Marrow Chromosome Aberration Test”, dated May 30, 2008.

⁵² For animals that showed a distinct positive result in fewer than 100 metaphases, 50 metaphases were examined (in accordance with OECD Guideline 475).

Table 10. Summary of chromosome aberration assay results for WAP

Study Groups (<i>n</i> = 5)	Metaphases	Aberrant Cells (Total/ % ± SD)	Mitotic Index
Negative Control, 24 h			
Male	500	3/ 0.6 ± 0.9	6.22
Female	500	3/ 0.6 ± 0.5	6.60
Positive Control, 24 h			
Male	350 ^a	138/ 39.4 ± 12.3 [*]	5.02
Female	300 ^b	94/ 31.3 ± 4.9 [*]	3.36
Treatment Group, 24 h			
Male	500	4/ 0.8 ± 0.4	8.08
Female	500	2/ 0.4 ± 0.5	8.26
Negative Control, 48 h			
Male	500	1/ 0.2 ± 1.6	9.36
Female	500	3/ 0.6 ± 0.5	8.74
Treatment Group, 48 h			
Male	500	3/ 0.6 ± 0.5	4.60 [*]
Female	500	1/ 0.2 ± 0.4	9.50

SD = Standard deviation; *n* = number.

^{*} *P* < 0.01, vs. corresponding 24-h negative control group.

^a Three mice (50 metaphases), two mice (100 metaphases).

^b Four mice (50 metaphases), one mouse (100 metaphases).

6.5.4. Clastogenicity assay for HLA F

The clastogenic potential of the closely related HLA F material was evaluated in an *in vivo* bone marrow chromosome aberration test in the mouse (Szabo *et al*, 2012; FDA, 2013) under the same conditions as those previously described for the testing of WAP. Prior to the main experiment, the MTD for the range-finding study defaulted, due to a lack of observed toxicity, to 2000 mg/kg bw, the highest dose evaluated for the assay. This dose was then selected as the maximum in the main study. Prepared in cottonseed oil one hour before treatment, HLA F at a dose of 2000 mg/kg bw was administered *via* gavage to ten male and ten female NMRI mice (7-13 weeks old) in a single application at study initiation. Negative control mice (*n* = 10/sex) were given the vehicle in similar volumes. Positive control animals (*n* = 5/sex) received a single dose of 40 mg/kg bw of CPA in physiological saline *via i.p.* injection. Bone marrow cells were harvested 24 hours and 48 hours post-administration for the treatment and negative control groups (*n* = 5/sex/group/time) and 24 hours post-administration for the positive control group. After bone marrow cells were fixed and stained, 100 metaphases⁵² were scored for cytogenic damage to determine the incidence of structural chromosomal aberrations. In addition, approximately 1000 cells were evaluated for cytotoxicity to determine the mitotic index. Although the male and female positive controls exhibited biologically and statistically significant increases in aberrant cell values (*P* < 0.01 for both vs. corresponding negative control), no dose dependent, biologically relevant or statistically significant increase in the incidence of aberrant cells or in the mitotic index was observed in the 24-hour or 48-hour dose groups compared to their corresponding negative controls. The findings (aberrant cell values and mitotic indices) for all groups except for those of the positive control were in the range of the historic negative controls. Under the conditions of this study, HLA F was not clastogenic.

In summary, neither WAP nor HLAf exhibited mutagenicity under the conditions of the bacterial reverse mutation assay or clastogenicity under the conditions of the *in vivo* bone marrow chromosome aberration assay. The genotoxicity studies are summarized in Table 11.

Table 11. Genotoxicity studies

Assay	Test system	Concentrations	Results	Reference
Bacterial reverse mutation assay	<i>S. typhimurium</i> TA98, TA100, TA1535, and TA1537; and <i>E. coli</i> WP2uvrA	Experiment I (standard plate incorporation): Up to 5000 µg/plate WAP in distilled water for all test strains and conditions (with and without S9 activation); Experiment II (pre-incubation): Up to 2500 µg/plate WAP in distilled water for all test strains and conditions.	Not mutagenic	Szabo <i>et al.</i> (2013)
Bacterial reverse mutation assay	<i>S. typhimurium</i> TA98, TA100, TA1535, and TA1537; and <i>E. coli</i> WP2uvrA	Experiment I (standard plate incorporation): Up to 5000 µg/plate HLAf in DMSO for all test strains and conditions (with and without S9 activation) Experiment II (pre-incubation): Up to 5000 µg/plate in DMSO for all test strains and conditions except for TA 1537 without S9 activation where 1000 µg/plate was highest dose due to cytotoxic effects.	Not mutagenic	Szabo <i>et al.</i> (2012); FDA (2013)
<i>In vivo</i> bone marrow chromosome aberration assay	NMRI Mouse (10M/10F/group)	0 and 2000 mg/kg WAP in physiological saline <i>via gavage</i> ; single dose	Not clastogenic	Szabo <i>et al.</i> (2013)
<i>In vivo</i> bone marrow chromosome aberration assay	NMRI Mouse (10M/10F/group)	0 and 2000 mg/kg bw HLAf in cottonseed oil <i>via gavage</i> ; single dose	Not clastogenic	Szabo <i>et al.</i> (2012); FDA (2013)

bw = body weight; DMSO = Dimethyl sulfoxide; F = Female; HLAf = High-lipid algalin flour; M = Male; WAP = Whole Algal Protein

6.6. Carcinogenesis

No studies addressing carcinogenicity were discovered in the scientific literature for *C. protothecoides* or *A. protothecoides*.

7. Human-Related Observations and Concerns

7.1. Organoleptic observations

Not intended to be consumed as a stand-alone food (and therefore, similar to flour, salt or cooking oil), WAP is a pale yellow to green ingredient³ that has a slight savory flavor. When used at its intended levels in food, taste of the final food is not adversely affected.

7.2. Allergenic potential

The evaluation of the allergenicity of the food ingredient rests on three pillars: (1) digestibility of the protein, (2) allergenic status of the protein source in the scientific literature, and (3) comparison of the amino acid sequences in the ingredient protein(s) with sequences in known allergenic proteins.

7.2.1. Digestibility

As *per* the ADME statement (Section 5.), the proteins found in WAP are expected to be digested, absorbed, metabolized and excreted through the same normal physiological processes by which plant materials common to the human diet are digested.

7.2.2. Allergenic status in the scientific literature

A search of the scientific literature did not indicate any association of allergy or allergic response to *C. protothecoides* or to any *Auxenochlorella* or *Prototheca* species. Published studies of allergy instead have primarily been limited to *Chlorella* species⁵³ *C. vulgaris*, *C. pyrenoidosa*, *C. saccharophila* and *C. homosphaera* (Tiberg *et al.*, 1990a; Tiberg *et al.*, 1990b; Genitsaris *et al.*, 2011). Although allergy to *Chlorella* has generally presented as hypersensitization resulting from exposure to algae-infested waters or respiratory allergy to airborne⁵⁴ algae (Bernstein and Safferman, 1966; Bernstein and Safferman, 1973; Tiberg *et al.*, 1995; Chrisostomou *et al.*, 2009; Genitsaris *et al.*, 2011), there is a published case study of oral allergy in an 11-year-old boy (Yim *et al.*, 2007). Three months into a daily regimen of *Chlorella* food supplements (200 mg/tablet, 10 tablets/day), acute tubulointerstitial nephritis⁵⁵ was diagnosed in the child. Renal function improved with cessation of the *Chlorella*⁵⁶ supplements and a six-month regimen of corticosteroids. A follow-up skin prick test (100 mg and 200 mg *Chlorella* in 5 ml distilled water) six months after steroid therapy failed to produce positive wheal reactions for the 100 mg or 200 mg test amounts. In a previous study by Tiberg and Einarsson (1989), the allergenic potencies of eight strains within four species of *Chlorella* (*C. vulgaris*, *C. homosphaera*, *C. saccharophila*, and *C. fusca*) were compared in an attempt to define a representative extract preparation for the genus. Due to the highly variable allergenic activity of the eight strains, the authors concluded that development of a representative extract preparation for the genus was unlikely and that “selection of a strain is a crucial step for the production of an allergen extract from *Chlorella*.” An evaluation of the scientific literature did not indicate any association of allergy or allergic response to any *C. protothecoides* or *A. protothecoides* species.

7.2.3. Comparison of potential allergens using translated genomic sequences

The Structural Database of Allergenic Proteins⁵⁷ (SDAP) administered by the University of Texas Medical Branch, Galveston, TX, is a worldwide database that contains all allergens

⁵³ When considering species identified in articles published prior to 2000, the possibility of reclassification is a concern especially for *C. vulgaris* and *C. pyrenoidosa* species. Verification against the culture collections of the depositories is recommended.

⁵⁴ Aerosolized or associated with house dust

⁵⁵ Tubulointerstitial nephritis is a common cause of acute renal failure from immune-mediated tubulointerstitial injury; hypersensitivity reactions can induce allergic response in the renal interstitium (Yim *et al.*, 2007).

⁵⁶ Composition of the tablets was not defined beyond the genus.

⁵⁷ <http://fermi.utmb.edu/SDAP/>

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from the IUIS (International Union of Immunological Societies) website,⁵⁸ supplemented with information from the scientific literature and from major sequence (SwissProt,⁵⁹ PIR,⁶⁰ and NCBI⁶¹) and structure (PDB⁶²) databases. To assess the allergenic potential of the *C. protothecoides* S106 proteome *in silico*, the basic local alignment search tool (BLAST) was used to query the entire amino acid sequence of the SDAP against the S106 genome, obtained *via* Next-Generation Sequencing with assembly of fragmented sequences. For comparison, the SDAP was also BLASTed against translations of the genomes of *Zea mays* (corn), *Lycopersicon esculentum* (tomato), and *Saccharomyces cerevisiae* (yeast). In addition, a translated, expressed sequence tag (EST) database from *Triticum aestivum*, (wheat) was also BLASTed. BLAST results are presented in a graphical format in Figure 3. As can be seen, similar to *S. cerevisiae* with 843 BLAST hits, the translated genome of *C. protothecoides* S106 has relatively few BLAST hits (1635 hits) against the SDAP allergens. Corn (12,879 hits), tomato (15,870 hits) and wheat (130,116 hits), on the other hand, have significantly greater numbers of hits. Thus, the well-known foods included in the evaluation (*i.e.*, corn, wheat, and tomato) have greater numbers of hits and with higher e-values (*i.e.*, more proteins that are more closely related to known allergenic epitopes), than *C. protothecoides* S106 does.

In summary, *in silico* analysis of the potential allergens in *C. protothecoides* S106 was provided by BLAST of the SDAP against five different translated genomes (corn, tomato, S106 and yeast) and one translated EST database (wheat). Genomes from three different food crops (*i.e.*, corn, tomato and wheat) had dramatically greater numbers of BLAST hits than did S106, indicating that, from an allergenicity standpoint, the protein component of whole *C. protothecoides* S106 biomass poses little risk as a potential human allergen.

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⁵⁸ <<http://www.allergen.org>>; site accessed April 2, 2014.

⁵⁹ SwissPROT = a protein database that contains sequences translated from the EMBL Nucleotide Sequence Database, prepared by the European Bioinformatics Institute

⁶⁰ PIR = Protein Information Resource

⁶¹ NCBI = National Center for Biotechnology Information

⁶² PDB = Brookhaven Protein Data Bank, a database of three-dimensional structures
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Figure 3. Number of putative allergen hits in various translated genomes or transcriptomes by BLAST with the SDAP

7.2.4. Supportive observations in humans

7.2.4.1. Human repeat-insult patch test with WAP

The potential for induced allergenic response to WAP³⁸ when consumed as a food has been investigated *via* dermal sensitization of human subjects (Szabo *et al*, 2013). The study design was in accordance with the applicable guidelines for the protection of human subjects for research as outlined in 21 CFR §50 and in accordance with the accepted standards for Good Clinical Practices (GCPs), International Conference on Harmonization (ICH Expert Working Group, 1996) and the standard practices of Thomas J. Stephens and Associates, Inc. (Carrollton, TX). Performance of a dermal patch test in humans, as a means of predicting food allergy by the occurrence of contact dermatitis, is a noninvasive approach that holds low risk for the safety of the participants. Written informed consent complying with 21 CFR §50.25 was obtained from each subject prior to study enrollment. A total of 130 healthy subjects enrolled in the Human

Repeat Insult Patch test (HRIPT) for WAP; 111 participants⁶³ (28 men, 83 women; average subject age of 45.06 ± 12.97 years) completed the study.

The HRIPT developed by Rizer and Nozawa (Trookman *et al.*, 2011) consists of two phases, an induction phase and a challenge phase. The three-week induction phase included nine patch periods. During each period, sufficient WAP test material to cover the test site (approximately 2 cm² in area) was applied under a patch to healthy skin on the upper back of each subject. The patch was removed 48 hours after application or about two hours before a study visit. Induction sites were graded 48 to 72 hours after each application, using a standardized skin irritation scale (Berger and Bowman, 1982). A minimum of twelve days after application of the last induction patch, challenge patches were applied to the original test site and to a naïve site, also on the upper back. These were removed 48 hours after application, or about two hours before the study visit. Challenge sites were graded 48 hours and 96 hours post-application. Occlusive patches (non-woven cotton pads covered by Blenderm tape and held securely to skin on all sides with a porous, hypoallergenic tape) were used for the first two patch periods in the induction phase; semi-occlusive patches (non-woven cotton pads covered and held securely to skin on all sides with a porous, hypoallergenic tape) were used for all later patch periods in the induction phase and for the entire challenge phase.

Of the 130 subjects who enrolled, one was discontinued for noncompliance, 16 for missing more than one study visit, and one withdrew for a cardiac condition unrelated to the test material. One additional subject withdrew after grading of the first occlusive patch determined moderate site reaction to the occluded test material. The site scored a “1” for erythema with evident papules. The subject withdrew from the study and the reaction was followed to resolution.

Because increasing instances of site irritation were broadly observed among the study participants at the second grading (23%; Table 12), semi-occlusive patches were substituted for occlusive patches for the remainder of the study. At the ninth (final) grading period in the induction phase, the test sites of three participants (2.7%) exhibited a mild erythema⁶⁴ (faint to definite pink) while the test sites of the other 108 participants (97.3%) presented with no visible erythema (Table 12). Changing the patch system from occlusive to semi-occlusive resolved the cumulative irritation evident in the early stages of the induction phase. The results of the challenge phase further demonstrated that the test material did not induce an allergic response in the study group. At the 96-h grading in the challenge phase, the original application site of one subject (0.9%) and naïve sites of two subjects (1.8%) exhibited mild erythema (Table 12). No other subjects demonstrated irritation.

Regarding the one subject withdrawn during the occlusive portion of the test (after the first patch period), the total evidence suggests that this individual is more likely to have experienced irritation, rather than allergic response. Although pre-existing skin sensitization is suggested by the rapidity (first of nine 48-h patch periods) and severity (papules) of the response, the response was not confirmed (*via* oral food challenge) to result from a true allergic reaction.

⁶³ The 111 subjects completing the study identified their ethnicity/race as follows: 11 African Americans (9.9%), 1 Asian (0.9%), 77 Caucasians (69.4%), 13 Hispanics (11.7%), 2 Native Americans (1.8%), 1 Pacific Islander (0.9%), and 6 subjects of mixed ethnicity (5.4%).

⁶⁴ Because redness can result from local irritation, mild erythema alone is not treated as potentially indicative of allergic response.

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Because erythema is known to result from local irritation (Cudowska and Kaczmariski, 2005), gradings of “1”, mild erythema without infiltration, are usually regarded as negative and a positive reaction is assessed only if erythema with infiltration or papules is present. Distinguishing irritation from allergy can be challenging for test materials with inherent irritant potential (McNamee *et al.*, 2008).⁶⁵ In addition, it is widely known that repeated exposure to a dermal irritant can result in a cumulative irritation reaction (Trookman *et al.*, 2011) and that the use of occlusive patching (an exaggerated exposure condition frequently used for this very reason), can exacerbate irritant effects (McNamee *et al.*, 2008).

In this study, WAP was applied as a dry solid to subjects’ backs under occlusive patching without dilution or extraction.⁶⁶ At the end of the second patch period (occlusive) nearly one-fourth (23%) of study volunteers exhibited some degree of patch site irritation; 23 participants presented with mild erythema (gradings of “1”), two individuals presented with mild erythema and one additional subject presented with papules only (no erythema). The use of semi-occlusive patching during the remainder of the study was found to resolve nearly all irritation concerns in the 111 remaining subjects for the remaining weeks of the study (Table 12). At the end of the induction phase, only three subjects presented with mild erythema at the application site. At the end of the challenge phase, one presented with mild erythema at the original site and two at the alternative (naïve) site. As an additional consideration, while atopic patch tests (APTs) have been shown to generate negative predictive values of about 90%⁶⁷ and have been found particularly useful in predicting late phase (T-cell-mediated) reactions⁶⁸ (Cudowska and Kaczmariski, 2005; Chung *et al.*, 2010) positive predictive values have been substantially lower (50% and 57% compared to DBPCFC⁶⁹ for milk and egg allergy, respectively, (Chung *et al.*, 2010); 29% for milk, casein, egg white and yolk (Peron *et al.*, 2011)). A tendency for false positives (20% compared to DBPCFC for milk allergy in older children; (Cudowska and Kaczmariski, 2005) is a recognized limiting factor of APT.

In summary, although mild-moderate irritation was identified in several subjects, WAP did not, under the conditions of this test, induce contact sensitization (allergic contact dermatitis) in any subject completing the study which suggests a low likelihood of food allergy.

⁶⁵ Wheat gluten is an example of a test material suspected of inducing false positive (irritant) reactions (Turjanmaa *et al.*, 2006; McNamee *et al.*, 2008).

⁶⁶ When a test material is a potential irritant, dilution or extraction is recommended as a means of decreasing the incidence of false positive responses (Turjanmaa *et al.*, 2006; McNamee *et al.*, 2008).

⁶⁷ 95.9% and 88.9% compared to double-blind placebo-controlled food challenge (DBPCFC) for milk and egg allergy, respectively (Chung *et al.*, 2010); and 90% for milk, casein, egg white and yolk (Peron *et al.*, 2011).

⁶⁸ In comparison, skin prick tests (SPT) and food-specific IgE antibodies *via* serological assay are indicative only of early (IgE-mediated) food allergy reaction (Cudowska and Kaczmariski, 2005).

⁶⁹ DBPCFC = Double-Blind Placebo-Controlled Food Challenge

Table 12. Score frequencies for WAP at each grading during the HRIPT induction and challenge phases

Score	Induction Phase									Challenge Phase			
	G1	G2	G3	G4	G5	G6	G7	G8	G9	48 O	96 O	48 A	96 A
0	97	85	105	109	106	110	106	107	108	107	110	104	109
0P	0	1	1	0	1	0	0	0	0	0	0	0	0
1	13	23	4	1	4	1	5	4	3	4	1	7	2
1P	1	2	1	1	0	0	0	0	0	0	0	0	0
Total	111	111	111	111	111	111	111	111	111	111	111	111	111

0 = No visible erythema; 1 = Mild erythema (faint pink to definite pink); 48 = 48-hour observation; 96 = 96-hour observation; A = Alternate (naive) site; G = Grading; HRIP = Human repeat-insult patch; O = Original site; P = papules; WAP = Whole Algal Protein

7.2.4.2. Human repeat-insult patch test with HLA F

The potential for induced allergic response to the closely related HLA F, when consumed as a food, has also been investigated *via* dermal sensitization in human subjects using the same approach as described for WAP (Szabo *et al.*, 2012; FDA, 2013). A total of 130 healthy subjects enrolled in the HRIPT for HLA F and 110 participants⁷⁰ (28 men, 82 women; average subject age of 45.01 ± 13.01 years) completed the study.

Of the 130 subjects who enrolled, one was discontinued for noncompliance, 16 for missing more than one study visit, and one withdrew for a cardiac condition unrelated to the test material. Two additional subjects were withdrawn after grading of the first and second occlusive patches determined moderate site reactions to the test material. One subject was scored during the first grading as having severe erythema (very intense redness) with edema (swelling), bullae⁷¹ and spreading at the application site; this subject withdrew from the study and the reaction was followed to resolution. The second subject was scored during the first grading as having severe edema with bullae at the application site. For this subject, an alternate site was used for the second patching and both the original and alternate sites were evaluated during the second grading. At the second grading, the original site scored “severe” for erythema with edema and papules⁷² and the alternate site scored as “severe” edema with bullae; the subject was then withdrawn and the reactions were followed to resolution.

Because increasing instances of site irritation were broadly observed among the study participants at the second grading (37%; Table 13), semi-occlusive patches were substituted for occlusive patches for the remainder of the study. At the ninth (final) grading in the induction phase, the test sites of four participants (3.6%) exhibited a mild erythema while the test sites of 106 participants (96.4%) presented with no visible erythema. After the change to semi-occlusive patching, no cumulative irritation was observed in the induction phase. The results of the challenge phase demonstrated that the test material did not induce an allergic response in the study group. At the 96-hour grading in the challenge phase, the original application sites of three

⁷⁰ The 110 subjects completing the study identified their ethnicity/race as follows: 11 African Americans (10.0%), 1 Asian (0.9%), 76 Caucasians (69.1%), 13 Hispanics (11.8%), 2 Native Americans (1.8%), 1 Pacific Islander (0.9%), and 6 subjects of mixed ethnicity (5.5%).

⁷¹ Vesicles (small, circumscribed elevations having translucent surfaces so that fluid is visible (*i.e.*, blister-like)) with a diameter > 0.5 cm; vesicles may coalesce to form one or a few large blisters that fill the patch site.

⁷² Small red solid elevations; when touched the affected surface has a granular feeling.

subjects (2.7%) and naïve site of one subject (0.9%) exhibited mild erythema. No other subjects demonstrated irritation.

Regarding the two subjects withdrawn during the occlusive portion of the test (one after the first and one after the second patch period), the total evidence suggests that these individuals are more likely to have experienced irritation, rather than allergic response. Although pre-existing skin sensitization was strongly suggested by the rapidity (first of nine 48-h patch periods), severity (edema and papules/bullae), and persistence of the responses (3-4 weeks to resolve) in both subjects, neither of the responses was confirmed (*via* oral food challenge) to result from a true allergic response.

In this study, HLA_F was applied as a dry solid to subjects' backs under occlusive patching without dilution or extraction.⁶⁶ At the end of the second patch period (occlusive) more than one-third (37%) of study volunteers exhibited some degree of patch site irritation (*i.e.*, erythema) with three individuals also presenting with papules and a fourth with edema. The use of semi-occlusive patching during the remainder of the study was found to resolve nearly all irritation concerns in the 110 remaining subjects for the remaining weeks of the study (Table 13). At the end of the induction phase, only four subjects presented with mild erythema at the application site. At the end of the challenge phase, three presented with mild erythema at the original site and only one at the alternative (naïve) site.

In summary, although mild-moderate irritation was identified in several subjects, HLA_F did not, under the conditions of this test, induce contact sensitization (allergic contact dermatitis) in any subject completing the study which suggests a low likelihood of food allergy.

Table 13. Score frequencies for HLA_F at each grading during the HRIPT induction and challenge phases

Score	Induction Phase									Challenge Phase			
	G1	G2	G3	G4	G5	G6	G7	G8	G9	48 O	96 O	48 A	96 A
0	81	68	99	106	98	106	106	105	106	106	107	103	109
0P	0	0	1	0	3	0	0	0	0	0	0	0	0
0R	0	1	0	0	0	0	0	0	0	0	0	0	0
1	27	36	9	3	9	4	4	3	4	4	3	7	1
1P	2	3	1	1	0	0	0	2	0	0	0	0	0
2	0	1	0	0	0	0	0	0	0	0	0	0	0
2E	0	1	0	0	0	0	0	0	0	0	0	0	0
Total	110	110	110	110	110	110	110	110	110	110	110	110	110

0 = No visible erythema; 1 = Mild erythema (faint pink to definite pink); 2 = Moderate erythema (definite redness); 48 = 48-hour observation; 96 = 96-hour observation; A = Alternate (naïve) site; E = Edema; G = Grading; HLA_F = High-lipid algalin flour; O = Original site; P = papules, R = Removal of the patch at other than the assigned time

7.3. Pathogenic potential

7.3.1. Pathogenic status in the scientific literature

Even though microalgae are ubiquitous organisms in the natural environment, algal infections are rare in humans and other mammals. Of those that do occur, the most common are protothecosis and chlorellosis, pseudofungal diseases caused, respectively, by opportunistic members of the achlorophyllous *Prototheca* spp. and green *Chlorella* spp. (Ramírez-Romero *et*

al., 2010). The two *Prototheca* species known to be disease agents are *P. wickerhamii*, the cause of most human protothecosis and *P. zopfii*, the usual causative agent in animals (Chandler *et al.*, 1978; Jagielski and Lagneau, 2007; Tap *et al.*, 2012). Thus far, the *Chlorella* species responsible for incidents of chlorellosis have not yet been identified. Chlorellosis is usually differentiated from protothecosis by the color of the lesions and/or affected organs. Protothecosis lesions tend to be ivory, white or cream-colored like the achlorophyllous causative organism and are difficult to distinguish in culture from common infectious yeast organisms (*i.e.*, *Candida* and *Cryptococcus*) (Tap *et al.*, 2012); chlorellosis lesions, on the other hand, are generally bright green or emerald in color (Chandler *et al.*, 1978).

Protothecosis, which is relatively rare, usually manifests in humans and animals as a localized (*i.e.*, cutaneous or articular) infection or, less commonly, as a disseminated systemic infection (Tap *et al.*, 2012). In the cow, clinical protothecosis usually presents as bovine mastitis. Although infection in cats and goats is also usually localized and cutaneous, infection in dogs can be severe, involving the eyes, brain, and internal organs (Jagielski and Lagneau, 2007; Satoh *et al.*, 2010). Protothecosis in humans most commonly presents as skin infection, usually from contamination of wounds or other breaks in the skin with dirty water (Torres *et al.*, 2003). Individuals who become ill with systemic infection are nearly always already debilitated or immunocompromised (Torres *et al.*, 2003; Jagielski and Lagneau, 2007; Ramírez-Romero *et al.*, 2010; Satoh *et al.*, 2010; Tap *et al.*, 2012). On a yearly basis, 2-5 cases of infection are typically reported, with 108 total cases of human protothecosis reported over a 25 year period (Krcméry Jr., 2000).

In comparison, chlorellosis has been reported in a variety of domestic and wild animals, including dogs, cattle, sheep, gazelle, dromedaries and beavers, but in only one human. The single reported incident of human infection presented as localized cutaneous lesions in surgical wounds exposed to river water (Ramírez-Romero *et al.*, 2010); the causative species was not identified. Although infection in the human patient was mild, chlorellosis can cause systemic disease in livestock (Chandler *et al.*, 1978; Ramírez-Romero *et al.*, 2010). Most bovine infections are mild (*i.e.*, infected lymph nodes found during slaughterhouse inspection); chlorellosis in sheep, however, is often enteric and frequently the cause of morbidity and mortality (Chandler *et al.*, 1978; Ramírez-Romero *et al.*, 2010). Although infection usually occurs through environmental contamination of open wounds, infection in animals has also been associated with drinking sewage water or stagnant water, or grazing pasture irrigated with untreated sewage water (Ramírez-Romero *et al.*, 2010).

7.3.2. Biosafety level designation

As previously defined in Section 2.A. 'Identity', the source microorganism for WAP, *C. protothecoides* S106 (UTEX 250, aka SAG 211-7C and CCAP 211/7C; original deposition CCAP (1952-5)), is appropriately considered a strain of *Auxenochlorella protothecoides* in addition to being a strain of *C. protothecoides*. Further, S106 has been demonstrated to be identical to the *Auxenochlorella protothecoides* type strain (*C. protothecoides* S485, aka UTEX 25; SAG 211-7A, CCAP211/7A, and ATCC 30407). All *C. protothecoides* (*A. protothecoides*) strains held at the ATCC in Rockville, Maryland, are preserved under BSL 1 conditions which indicate the microorganisms are not recognized to cause disease in immunocompetent adult humans.⁹

7.3.3. Corroborative pathogenicity study

As corroborative evidence supporting a BSL 1 designation for the *C. protothecoides* S106 source organism utilized in the production of WAP, a pathogenicity study designed to monitor the possible survival or propagation of Microbial Pest Control Agents (MPCA) was conducted using the rat model (Solazyme Roquette Nutritionals, 2012a). The in-life dosing and tissue collection portion of the study was based on US FDA Toxicological Principles for the Safety Assessment of Food Ingredients, Redbook 2000, IV.C. 3a: *Short-Term Toxicity Studies with Rodents* (2003) and US EPA Health Effects Test Guidelines, OPPTS⁷³ 885.3050, Acute Oral Toxicity/Pathogenicity (1996); and conducted under GLP.⁷⁴ The tissue culture and analysis portion of the MPCA study was also conducted under GLP⁴⁹ conditions and in accordance with the EPA Microbial Pesticide Test Guidelines, OPPTS 885.3050, “Acute Oral Toxicity/Pathogenicity”, US Environmental Protection Agency, Prevention, Pesticides and Toxic Substances (7101). EPA 712-C-96-315, February 1996.

In order to evaluate whether or not *C. protothecoides* S106 microalgae could survive or propagate in the rat, 32 HSD:SD[®] rats (12 weeks old; mean weight $\pm 20\%$ for each sex) were randomly assigned to either the treatment ($n = 12/\text{sex}$) or vehicle control ($n = 4/\text{sex}$) group. Either live microalgal cells ($\sim 2.5 \times 10^8$ CFU⁷⁵/rat) in 2 ml vehicle (Defined EBO2⁷⁶ minus Co^{2+} and nitrogen) or 2 ml vehicle alone was administered *via* oral gavage to each rat as an acute dose. All treatment animals (24 rats) and half of the vehicle control animals (2 males, 2 females) were housed in one room. The remaining vehicle control animals (2 males, 2 females) were housed in a separate room. All rats were provided 2016CM Harlan Teklad Global Rodent Diet[®] (Harlan Teklad, Inc., Indianapolis, IN) and filtered tap water *ad libitum*. All rats were monitored for viability twice *per* day with cage-side observations twice the first day and once *per* day thereafter. Body weights for control rats were recorded during acclimation and on Days 1, 3, 7, 14 and 21, with terminal weights recorded on Day 22. Body weights for all treatment rats were recorded during acclimation and on Day 1, with terminal weights recorded for the first subgroup ($n = 3/\text{sex}$) on Day 4. Body weights for the second subgroup ($n = 3/\text{sex}$) were recorded on Day 7; terminal weights were recorded on Day 8. Third subgroup body weights ($n = 3/\text{sex}$) were recorded on Days 7 and 14; terminal weights were recorded on Day 15. Fourth subgroup body weights ($n = 3/\text{sex}$) were recorded on Days 7, 14 and 21; terminal weights were recorded on Day 22. Tissue,⁷⁷ blood (*i.e.*, EDTA⁷⁸/citrate buffered blood and plasma), and fecal samples were collected over a period of three weeks post-dosing (Days 3 (feces only), 4, 8, 15 and 22), transferred (under controlled conditions of 2 – 8 °C) from PSL (Dayton, NJ) to BSL Bioservice (Planegg, Germany) for analysis.

No mortalities occurred during the in-life portion of the study. No effect in clinical signs, mean body weight or mean body weight gain were found in the treatment groups, compared to

⁷³ OPPTS = Office of Prevention, Pesticides, and Toxic Substances

⁷⁴ US EPA GLP: Toxic Substances Control Act (TSCA): 40 CFR 792, 1989 and US FDA GLP: 21 CFR 58, 1987

⁷⁵ CFU = Colony Forming Units

⁷⁶ Defined EB02 = A proprietary media formulation developed by Solazyme containing: 3 g/L K_2HPO_4 , 5.66 g/L $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 1.2 g/L citric acid monohydrate, 1 g/L $(\text{NH}_4)_2\text{SO}_4$, 0.23 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.03 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1 mL trace elements minus Co^{2+} , 0.225 mL/L antifoam (Sigma 204), 1.5 mL DAS vitamins, glucose to 40 g/L. Defined EB02 minus Co^{2+} and nitrogen was prepared as above except ammonium sulfate was omitted.

⁷⁷ Brain, lung, liver, spleen, kidney, and lymph nodes

⁷⁸ EDTA = Ethylenediaminetetraacetic acid

the control groups. In addition, no differences were identified in any monitored parameter between the control groups (housed with or isolated from the test group). Although two incidental findings (a small-appearing kidney in one control male and one treatment female) were reported at necropsy, no macroscopic differences were observed among the groups.

Tissue, blood, plasma, and fecal samples were analyzed for the presence of *C. protothecoides* cells using a surface spread method with plate counting.⁷⁹ All sample and control plates were prepared in triplicate; each plate was analyzed in duplicate. After incubation (3 – 8 days at 30 ± 2 °C), all plates prepared from tissue, blood, and feces samples were analyzed by plate counting for CFU/plate of the microalgae. Negative control (basal medium) and positive control plates (basal medium inoculated with 1:100, 1:1000 and 10,000 dilutions of 2-4 day old *C. protothecoides* S106 cultures) were analyzed in the same manner. Plate counting determined that algal colony counts were below the limits of detection⁸⁰ for all treatment and control tissue, blood (except for unreadable plates from three control animal samples), plasma, and feces samples for all collection days. Of all plates prepared for analysis, one sample from each of three control animals (two female Day 22 heparinized bloods and one male Day 22 brain) could not be read due to overgrowth by molds and red-colony bacteria. Although the analyses of nearly all fecal samples⁸¹ from all days were also affected by rapid overgrowth of molds, each of the fecal plates could still be analyzed and each reported an algal count below the limit of detection. No presence or growth of *C. protothecoides* S106 was observed on the negative control plates (< 1 CFU/ml negative control inoculum, equivalent to < 1 CFU/plate). The results of the positive controls, expressed as CFU/ml positive control inoculum, (equivalent to CFU/plate), were directly comparable to the CFU/mL of the 2 – 4 day *C. protothecoides* S106 cultures.

In summary, one incident of chlorellosis has been documented in a human. The infection arose from the exposure of surgical wounds to contaminated water and was limited to cutaneous lesions. Because lesional organisms are rarely cultured for genotyping, there is no data that addresses whether or not *C. protothecoides* was the opportunistic *Chlorella* species in the human patient or in the animals that are occasionally infected.

Because only one case of chlorellosis in humans has been reported in the scientific literature, it is highly unlikely that *C. protothecoides* has or will be the cause of human disease. In addition, *C. protothecoides* and *A. protothecoides* spp. cultures have a BSL 1 rating. Further, as corroborative evidence supporting the limited findings of one incident of opportunistic infection in one human in the scientific literature (from an unknown *Chlorella* spp.) and the BSL 1 designation for *C. protothecoides* and *A. protothecoides* spp., no adverse or toxic effect related to treatment was observed in any rat in the pathogenicity study. No viable count of *C. protothecoides* S106 was detected in any examined tissues or fluids collected during a three-

⁷⁹ Prior to initiation of the pathogenicity study in the rats, the surface spread method was validated for quantification of *C. protothecoides* S106 spiked into tissue samples (brain, lung, liver, spleen, kidney and lymph nodes) and into blood and feces of control animals (Solazyme Roquette Nutritionals, 2012b). The surface spread method is the method of choice for quantification of colony forming units of aerobic microorganisms (USP <61>).

⁸⁰ All limits of detection (LODs) were normalized to the volume (blood) or weights (tissues and feces) of the samples: < 5 CFU/ml plasma and heparinized blood; < 14 – < 20 CFU/g brain; < 15 – < 19 CFU/g lung; < 14 – < 19 CFU/g liver; < 14 – < 20 CFU/g spleen; < 14 – < 19 CFU/g kidney; < 14 – < 18 CFU/g lymph node; < 14 – < 31 CFU/g feces for 29 of 32 feces samples with the LOD for one sample at < 54 CFU/g, for a second at < 67 CFU/g, and for a third at < 115 CFU/g (all extremely small, highly diluted samples from Day 15).

⁸¹ All treatment fecal samples from Days 4, 8, 15, and 22; seven out of eight control fecal samples from Day 22.

week post-treatment period after the administration of the live algal culture. Under the conditions of the pathogenicity study in rodents, *C. protothecoides* S106 was not acutely toxic, pathogenic or toxigenic. Taken together with the record in the scientific literature and the nonpathogenic BSL rating, it is highly unlikely that consumption of *C. protothecoides* S106 or HLAf derived from *C. protothecoides* S106 would result in a pathogenic incident.

7.4. Photosensitive dermatitis

In 1977, at least 23 residents of Tokyo were diagnosed with photosensitive dermatitis⁸² (Tamura *et al.*, 1979; Jassby, 1988). Investigation revealed that each patient had consumed the same brand of *Chlorella* tablet (“Kenbi Chlorella”) and, further, that the tablets contained PheideA and its ester in high quantities (up to 8.2 mg/g total PheideA (Jassby, 1988)). A subsequent feeding trial in mice confirmed the association when administration of suspect tablets induced the phototoxic response and administration of *Chlorella* tablets having lower concentrations of Pheides (< 10% of suspect concentrations) did not. The researchers were also able to establish a linear dose-response relationship between inflammation severity and the Pheide content of the administered tablets (Jitsukawa *et al.*, 1984; Jassby, 1988).

Pheophorbide-associated phototoxicity occurs after ingested Pheides enter the circulatory system and distribute throughout the body. When Pheides that have deposited in the skin or other near-surface tissues are exposed to light, oxygen is generated and the fatty acids of nearby cell membranes are subsequently oxidized which leads to cell rupture, damage to dermal capillaries and escalating inflammation (Jitsukawa *et al.*, 1984; Jassby, 1988). A change in the manufacturing process (the use of ethanol during granule formation of the dried *Chlorella* prior to pelleting) was determined to have been the cause of the high PheideA concentrations in the Tokyo incident (Jitsukawa *et al.*, 1984). The concentrations of existing and potential total PheideA permitted in algae preparations were limited, respectively, to 0.8 mg/g and < 1.2 mg/g by the Japanese Public Health Ministry in 1981 (Jassby, 1988).

Because Pheides are natural degradation products of chlorophylls (Chls), they are present in all photosynthetic plants. In nature, Chl catabolites, including the phototoxic Pheides, typically increase in concentration during leaf senescence, fruit ripening or other incidents of “de-greening” (Jassby, 1988; Hörtensteiner *et al.*, 2000). During “de-greening” events, Pheides form from Chl through the step-wise loss of the phytol residue *via* chlorophyllase followed by the loss of the central magnesium atom *via* magnesium dechelataase (Hörtensteiner, 1999; Hörtensteiner *et al.*, 2000). As mentioned previously, certain manufacturing steps can affect Pheide formation in Chl-containing microalgae products including the use of ethanol, acetone or methanol during processing which can enhance chlorophyllase activity and production of Pheides. On the other hand, heating Chl-containing materials to 100 °C for three minutes is sufficient to inactivate the chlorophyllase enzyme (Jassby, 1988). To ensure against Pheide formation in preparations of microalgae that contain Chl, enzyme inactivation is often combined with an avoidance of processing steps using ethanol, acetone or methanol (Jassby, 1988).

The mechanism of Chl breakdown has been extensively studied in *C. protothecoides* (Hörtensteiner *et al.*, 1998; Hörtensteiner, 1999; Hörtensteiner *et al.*, 2000), the bleaching or de-greening of which under high glucose/nitrogen deficient conditions has been compared to the

⁸² Skin inflammations characterized by rash and itchiness that develop on exposure to light. Usual presentation is cutaneous lesions on the face and dorsa (backs) of the hands. In severe cases, lesions necrotize and scar.

Chl catabolism of senescing plants (Hörtensteiner *et al.*, 2000), albeit with some substantial differences:

- (1) Higher plants contain exclusively Chl-a. Although Chl-a is the major Chl in all algae, species within Division Chlorophyta, such as *C. protothecoides* also contain Chl-b (Becker, 1994).
- (2) Because *C. protothecoides* contains both Chl-a and Chl-b, colored catabolites of Chl degradation form for both Chls (Hörtensteiner *et al.*, 1998).
- (3) In higher plant species the majority of intermediate and final Chl catabolites are colorless, but in *C. protothecoides*, the main intermediate catabolites are the green-colored Pheides (PheideA and PheideB) with the final catabolites being the red-colored Red Chl catabolites (RCC-a and RCC-b) (Hörtensteiner *et al.*, 1998; Hörtensteiner, 1999).
- (4) After rapid formation from the green-colored Pheides in a two-step ring-opening of the porphyrin macrocycle (Hörtensteiner, 1999; Hörtensteiner *et al.*, 2000), algal RCCs are excreted into the medium. In higher plants, RCC-a (there is no RCC-b) is further degraded into a variety of Fluorescent and Nonfluorescent Chlorophyll Catabolites (FCCs and NCCs) (Hörtensteiner, 1999) which are then deposited into the vacuoles of senescent mesophyll cells (Hörtensteiner *et al.*, 1998).

Ordinary commercial *Chlorella* products generally contain 2-3% dry weight Chl content (Jassby, 1988). *C. protothecoides* Krüger ACC⁸³ 25, however, has been shown to have no detectable Chl and diminished chloroplast structures⁸⁴ when 'glucose-bleached' in high glucose/low nitrogen medium (Grant and Hommersand, 1974). WAP is generated from *C. protothecoides* S106 (UTEX 250), a strain that is identical to *C. protothecoides* UTEX 25 (S485) which is also known as *C. protothecoides* Krüger ACC No. 25.⁸⁵ When this strain is grown in (and isolated from) a nitrogen-limited medium – as is done in the manufacture of the closely related HLAf, Chl is absent and chloroplasts are minimized (Wu, *et al.*, 1994; Szabo *et al.*, 2012). Under the medium conditions of the WAP manufacturing process (which are not nitrogen-limited), Chl and, presumably, chloroplasts are present in the algal cells and subsequent WAP ingredient. To inactivate the chlorophyllase enzyme, the WAP manufacturing process includes a pasteurization step early in the recovery process, in addition to drying steps (see Section 2.D. 'Method of Manufacture of WAP', Figure 2); moreover no process steps involve solvents associated with increased production of Pheides. Consistent with these safeguards, total PheideA concentration has been measured in WAP at 0.0334 mg/g (FDA, 2012; Szabo *et al.*, 2013), a concentration considerably lower than the limit of < 1.2 mg/g established by the Japanese Public Health Ministry in 1981 (Becker, 1994; FDA, 2012).

⁸³ Algal Culture Collection at Indiana University, Bloomington, IN.

⁸⁴ As measured by absorbance, Chl returns to normal levels within 48 hr of providing a 'greening' medium (little/no glucose, high nitrogen) (Grant and Hommersand, 1974).

⁸⁵ UTEX 25 was initially designated as ACC No. 25 when the collection was held by Indiana University. In 1976 the ACC at Indiana University was transferred to the University of Texas and became the core of the UTEX collection. <<http://www.sbs.utexas.edu/utex/insideUtex.aspx>>; site accessed April 1, 2014.

8. Evaluation

WAP is a whole algal protein composed of the dried biomass of the microalgae *Chlorella protothecoides* S106. WAP can be used as a protein source, analogous to soy- and animal-based proteins, in a variety of foods.¹

Although statistically significant effects were noted for several endpoints in a published 28-day short-term repeated-dose study in rats, these were not found to be related to administration of a ground yellow, high-lipid *C. protothecoides* biomass material. Under the conditions of the study, the NOAEL for the biomass material in the diet was 100,000 ppm, the highest dietary concentration provided, which corresponds to a dietary NOAEL of 7557 mg/kg bw/day in male rats and 8068 mg/kg bw/day in female rats. In corroborative support of these findings, an unpublished 28-day short-term repeated-dose study in hamsters demonstrated that consumption of *C. protothecoides* at up to ~6000 mg/kg bw/day was safe.

In a published 13-week subchronic toxicity study examining WAP in rats, statistically significant effects were identified for several endpoints, but none were attributable to ingestion of WAP because the changes were incidental or noted only sporadically, were not accompanied by corresponding clinical or histopathologic changes, did not demonstrate a dose-dependent relationship, and/or were also observed in the control group. Under the conditions of this study, the NOAEL for WAP in the diet was 100,000 ppm, the highest dietary concentration provided, which corresponds to a dietary NOAEL of 4805 mg/kg bw/day in male rats and 5518 mg/kg bw/day in female rats. In a published 13-week subchronic toxicity study examining the closely related HLAf in rats, statistically significant effects were also reported for several endpoints, but were not attributable to ingestion of HLAf because the changes were within the ranges historically observed in the age and strain of rats used in this study, did not demonstrate a dose-dependent relationship, were noted only sporadically, and/or were also observed in the control group. Under the conditions of this study, the NOAEL for HLAf in the diet was 100,000 ppm, the highest dietary concentration provided, which corresponds to a dietary NOAEL of 4807 mg/kg bw/day in male rats and 5366 mg/kg bw/day in female rats.

The results of the bacterial reverse mutation and *in vivo* bone marrow chromosome aberration assays indicate that WAP is neither mutagenic nor clastogenic. The results of bacterial reverse mutation and *in vivo* bone marrow chromosome aberration assays also indicated that the closely related HLAf material was not mutagenic or clastogenic.

In a study exploring the effect of consuming live cells or the isolated cell walls of *C. protothecoides* 902 (20-23 g/kg bw/day) on the $t_{1/2}$ of chlordecone in ¹⁴C-chlordecone-treated rats, no adverse effects associated with consumption of the living cells or the cell walls of the algae were reported by the authors.

WAP has a low likelihood of being a food allergen. WAP proteins are expected to be digested, absorbed, metabolized and excreted through the same normal physiological processes by which plant materials common to the human diet are digested. In addition, a search of the scientific literature did not indicate any association of allergy or allergic response to *C. protothecoides* or to any *Auxenochlorella* or *Prototheca* species. Also, a BLAST of the translated genome for *C. protothecoides* S106 garnered few hits, indicating little risk of S106 being a human allergen. Finally, under the conditions of the HRIPT conducted in healthy male and

female human subjects, WAP did not induce contact sensitization (allergic contact dermatitis) in any subject completing the study, although mild-moderate irritation (but not allergic response) was identified in several subjects. The results of the study therefore indicate a low likelihood of food allergy. In addition, under the conditions of another HRIPT conducted in healthy male and female human subjects, the closely related HLAF also did not induce contact sensitization in any subject completing the study, although mild-moderate irritation (but not allergic response) was identified in several subjects. The results of this study also indicate a low likelihood of food allergy. Taken together, WAP is highly unlikely to be a food allergen.

Human infection by *C. protothecoides* is also extremely unlikely. Only one case of human chlorellosis has been documented in the literature and this infection was localized and cutaneous, the result of surgical wounds exposed to contaminated water. In addition, *C. protothecoides* and *A. protothecoides* strains are categorized as nonpathogenic BSL 1 microorganisms in respected culture collections such as ATCC. Corroboratively, no viable counts of *C. protothecoides* S106 were detected in any of the examined tissues from a three-week pathogenicity study in which rats were acutely dosed with live *C. protothecoides* S106 ($> 2.5 \times 10^8$ CFU/rat). Under the conditions of this study, *C. protothecoides* S106 was not pathogenic.

Assay of Solazyme's high protein *C. protothecoides* biomass reported a PheideA concentration of 0.0334 mg/g, a concentration that is below the permitted limit of 0.8 mg/g PheideA in algal preparations and well below the limit of < 1.2 mg/g for total potential PheideA imposed by the Japanese Public Health Ministry. The results of the conducted assay indicate that insufficient PheideA is present in WAP for photosensitivity to occur.

The NOAEL for WAP in a 13-week subchronic toxicity study in male and female rats was 100,000 ppm, the highest dietary concentration provided. The dietary NOAEL for WAP in male and female rats was 4805 and 5518 mg/kg bw/day, respectively (equivalent to 288,300 mg/day in a 60 kg human).

The estimated daily intake of WAP is based on the 90th percentile WAP consumption levels from foods supplemented with WAP; the estimated daily intake of WAP when added to foods was determined to be 5.56 g/day. Thus, the potential theoretical maximum WAP consumption at the 90th percentile may reach 5.56 g/day. This theoretical intake level represents a conservative estimate because it is unlikely that an individual would consume WAP from conventional foods at the 90th percentile level.

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9. References

- Becker, E. W. (1994) Chemical composition. In *Microalgae: Biotechnology and Microbiology*. Cambridge University Press, Cambridge, England. p. 177-249.
- Becker, E. W. (2007) Micro-algae as a source of protein. *Biotechnology Advances* 25:207-210.
- Berger, R. S. and Bowman, J. P. (1982) A reappraisal of the 21-day cumulative irritation test in man. *Journal of Toxicology - Cutaneous and Ocular Toxicology* 1:109-115.
- Bernstein, I. L. and Safferman, R. S. (1966) Sensitivity of skin and bronchial mucosa to green algae. *Journal of Allergy* 38:166-173.
- Bernstein, I. L. and Safferman, R. (1973) Clinical sensitivity to green algae demonstrated by nasal challenge and *in vitro* tests of immediate hypersensitivity. *The Journal of Allergy and Clinical Immunology* 51:22-28.
- Brown, M. R. and Jeffery, S. W. (1992) Biochemical composition of microalgae from the green algal classes Chlorophyceae and Prasinophyceae. 1. amino acids sugars and pigments. *Journal of Experimental Marine Biology and Ecology* 161:91-113.
- Chandler, F. W., Kaplan, W. and Callaway, C. S. (1978) Differentiation between *Prototheca* and morphologically similar green algae in tissue. *Archives of Pathology and Laboratory Medicine* 102:353-356.
- Chrisostomou, A., Moustaka-Gouni, M., Sgardelis, S. and Lanaras, T. (2009) Air-dispersed phytoplankton in a mediterranean river-reservoir system (aliakmon-polyphytos, Greece). *Journal of Plankton Research* 31:877-884.
- Chung, B. Y., Kim, H. O., Park, C. W. and Lee, C. H. (2010) Diagnostic usefulness of the serum-specific IgE, the skin prick test and the atopy patch test compared with that of the oral food challenge test. *Annals of Dermatology* 22:404-411.
- Conte, M. V. and Pore, R. S. (1973) Taxonomic implications of *Prototheca* and *Chlorella* cell wall polysaccharide characterization. *Archives of Microbiology* 92:227.
- Cudowska, B. and Kaczmarek, M. (2005) Atopy patch test in the diagnosis of food allergy in children with atopic eczema dermatitis syndrome. *Roczniki Akademii Medycznej w Białymstoku (1995)* 50:261-267.
- Day, A. G., Brinkmann, D., Franklin, S., Espina, K., Rudenko, G., Roberts, A. and Howse, K. S. (2009) Safety evaluation of a high-lipid algal biomass from *Chlorella protothecoides*. *Regulatory Toxicology and Pharmacology* 55:166-180.
- Dwyer, J., Picciano, M. F. and Raiten, D. J. (2003) Estimation of usual intakes: what we eat in America-NHANES. *The Journal of Nutrition* 133:609S-623S.
- FDA (2012) GRN 384. GRAS Notification of "Algal oil derived from *Chlorella protothecoides* strain S106 (Cp algal oil)" <<<http://www.accessdata.fda.gov/scripts/fcn/fcnNavigation.cfm?rpt=grasListing>>>, site visited April 1, 2014.

- FDA (2013) GRN 469. GRAS Notification of “*Chlorella protothecoides* strain S106 flour with 40-70% lipid (algal flour)” <<<http://www.accessdata.fda.gov/scripts/fcn/fcnNavigation.cfm?rpt=grasListing>>>, site visited April 1, 2014.
- Ferris, M. J., Sheehan, K. B., Köhl, M., Cooksey, K., Wigglesworth-Cooksey, B., Harvey, R. and Henson, J. M. (2005) Algal species and light microenvironment in a low-pH, geothermal microbial mat community. *Applied and Environmental Microbiology* 71:7164-7171.
- Genitsaris, S., Kormas, K. A. and Moustaka-Gouni, M. (2011) Airborne algae and cyanobacteria: Occurrence and related health effects. *Frontiers in Bioscience* E3:772-787.
- Grant, N. G. and Hommersand, M. H. (1974) The respiratory chain of *Chlorella protothecoides*. *Plant Physiology* 54:50-56.
- Harding, S. and Jones, P. (2008) Biological Evidence for the Insulin Independent Lowering of Blood Glucose by *Chlorella protothecoides*. Unpublished Report. p. 1-6.
- Hörtensteiner, S., Wüthrich, K. L., Matile, P., Ongania, K. H. and Kräutler, B. (1998) The key step in chlorophyll breakdown in higher plants. Cleavage of pheophorbide a macrocycle by a monooxygenase. *Journal of Biological Chemistry* 273:15335-15339.
- Hörtensteiner, S. (1999) Chlorophyll breakdown in higher plants and algae. *Cellular and Molecular Life Sciences* 56:330-347.
- Hörtensteiner, S., Chinner, J., Matile, P., Thomas, H. and Donnison, I. S. (2000) Chlorophyll breakdown in *Chlorella protothecoides*: characterization of degreening and cloning of degreening-related genes. *Plant Molecular Biology* 42:439-450.
- Huss, V. A. R., Frank, C., Hartmann, E. C., Hirmer, M., Kloboucek, A., Seidel, B. M., Wenzeler, P. and Kessler, E. (1999) Biochemical taxonomy and molecular phylogeny of the genus *Chlorella sensu lato* (Chlorophyta). *Journal of Phycology* 35:587-598.
- Huss, V. A., Ciniglia, C., Cennamo, P., Cozzolino, S., Pinto, G. and Pollio, A. (2002) Phylogenetic relationships and taxonomic position of *Chlorella*-like isolates from low pH environments (pH < 3.0). *BMC Evolutionary Biology* 2:13.
- ICH Expert Working Group (1996) ICH Harmonized Tripartite Guideline. Guideline for Good Clinical Practice E6(R1). International Conference of Harmonization (ICH). p. 1-53.
- Jagielski, T. and Lagneau, P. E. (2007) Protothecosis. A pseudofungal infection. *Journal de Mycologie Medicale* 17:261-270.
- Jassby, A. (1988) Some public health aspects of microalgal products. In *Algae and Human Affairs*. (C. A. Lembi and J. R. Waaland, Eds.) Cambridge University Press, p. 181-202.
- Jitsukawa, K., Suizu, R. and Hidano, A. (1984) *Chlorella* photosensitization. New phytophotodermatitis. *International Journal of Dermatology* 23:263-268.
- Kay, R. A. (1991) Microalgae as food and supplement. *Critical Reviews in Food Science and Nutrition* 30:555-573.
- Kessler, E. and Huss, A. R. (1992) Comparative physiology and biochemistry and taxonomic assignment of the *Chlorella* (Chlorophyceae) strains of the Culture Collection of the University of Texas at Austin. *Journal of Phycology* 28:550-553.

- Kreméry Jr., V. (2000) Systemic chlorellosis, an emerging infection in humans caused by algae. *International Journal of Antimicrobial Agents* 15:235-237.
- Lobeau, S. (2013) Whole Algalin Protein = Not Novel in Australia. (Personal Communication).
- McNamee, P. M., Api, A. M., Basketter, D. A., Frank Gerberick, G., Gilpin, D. A., Hall, B. M., Jowsey, I. and Robinson, M. K. (2008) A review of critical factors in the conduct and interpretation of the human repeat insult patch test. *Regulatory Toxicology and Pharmacology* 52:24-34.
- Peron, A., Tenconi, R., Leone, M., Macellaro, P., Ceriani, E. and D'Arcais, A. F. (2011) Negative atopy patch test and negative skin prick test reduce the need for oral food challenge in children with atopic dermatitis. *Pediatric, Allergy, Immunology, and Pulmonology* 24:107-112.
- Pore, R. S. (1984) Detoxification of chlordecone poisoned rats with chlorella and chlorella derived sporopollenin. *Drug and Chemical Toxicology* 7:57-71.
- Ramírez-Romero, R., Rodríguez-Tovar, L. E., Nevárez-Garza, A. M. and López, A. (2010) *Chlorella* infection in a sheep in Mexico and minireview of published reports from humans and domestic animals. *Mycopathologia* 169:461-466.
- Ravishankar, G. A., Sarada, R., Kamath, B. S. and Namitha, K. K. (2006) Food applications of algae. In *Food Biotechnology*. (K. Shetty, G. Paliyath, A. Pometto and R. E. Levin, Eds.) 2nd Edition. CRC Press, Boca Raton, FL. p. 491-521.
- Rawat, S., Agarwal, P. K., Choudhary, D. K. and Johri, B. N. (2005) Microbial diversity and community dynamics of mushroom compost ecosystem. In *Microbial Diversity: Current perspectives and potential applications*. (T. Satyanarayana and B. N. Johri, Eds.) I. K. International Publishing House, New Delhi, India.
- Robinson, R. K. and Guzman-Juarez, M. (1978) The nutritional potential of the algae. *Plant Foods for Man* 2:195-202.
- Satoh, K., Ooe, K., Nagayama, H. and Makimura, K. (2010) *Prototheca cutis* sp. nov., a newly discovered pathogen of protothecosis isolated from inflamed human skin. *International Journal of Systematic and Evolutionary Microbiology* 60:1236-1240.
- Solazyme Roquette Nutritionals, L. (2012a) Whole Algal Cell Culture: An Acute Oral Toxicity Study In Rats. April 5, 2012. Unpublished report. Performing Laboratory: Product Safety Labs. Report Number: 33135 p. 1-139.
- Solazyme Roquette Nutritionals, L. (2012b) Validation of the Viable Count Determination in Rat Matrices with Chlorella Protothecoides. January 11, 2012. Unpublished Report. Performing Lab: BSL Bioservice Report Number: 113723 p. 1-23.
- Stackebrandt, E. and Goebel, B. M. (1994) Taxonomic note: A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *International Journal of Systematic Bacteriology* 44:846-849.

- Szabo, N., Matulka, R. A., Kiss, L. and Licari, P. (2012) Safety evaluation of a high lipid whole algalin flour (WAF) from *Chlorella protothecoides*. *Regulatory Toxicology and Pharmacology* 63:155-165.
- Szabo, N. J., Matulka, E. A. and Chan, T. (2013) Safety studies of whole algalin protein (WAP) from *Chlorella protothecoides*. *Food and Chemical Toxicology* 59:34-35.
- Tamura, Y., Maki, T. and Shimamura, Y. (1979) Causal substances of photosensitivity dermatitis due to chlorella ingestion (Hygienic studies on Chlorella. I). *Journal of the Food Hygienic Society of Japan* 20:173-180.
- Tap, R. M., Sabaratnam, P., Salleh, M. A., Razak, M. F. and Ahmad, N. (2012) Characterization of *Prototheca wickerhamii* isolated from disseminated algaemia of kidney transplant patient from Malaysia. *Mycopathologia* 173:173-178.
- Tartar, A., Boucias, D. G., Becnel, J. and Adams, B. J. (2003) Comparison of plastid 16S rRNA (rrn16) genes from *Helicosporidium* spp.: evidence supporting the reclassification of *Helicosporidia* as green algae (Chlorophyta). *International Journal of Systematic and Evolutionary Microbiology* 53:1719-1723.
- Tiberg, E. and Einarsson, R. (1989) Variability of allergenicity in eight strains of the green algal genus *Chlorella*. *International Archives of Allergy and Applied Immunology* 90:301-306.
- Tiberg, E., Rolfsen, W. and Einarsson, R. (1990a) Preparation of allergen extracts from the green alga *Chlorella*. Studies of growth variation, batch variation, and partial purification. *International Archives of Allergy and Applied Immunology* 92:23-29.
- Tiberg, E., Rolfsen, W., Einarsson, R. and Dreborg, S. (1990b) Detection of *Chlorella*-specific IgE in mould-sensitized children. *Allergy: European Journal of Allergy and Clinical Immunology* 45:481-486.
- Tiberg, E., Dreborg, S. and Björkstén, B. (1995) Allergy to green algae (*Chlorella*) among children. *Journal of Allergy and Clinical Immunology* 96:257-259.
- TNO (2012) TNO-Memorandum to Solazyme Roquette Nutritionals regarding TNO V9461 Final Report Assessment on the regulatory status of *Chlorella protothecoides* with regard to EU Regulation 258/97 EC on Novel Foods, dated January 2012. Report Number: V9461 p. 1-11.
- Tokusoglu, O. and Unal, M. K. (2003) Biomass nutrient profiles of three microalgae: *Spirulina platensis*, *Chlorella vulgaris*, and *Isochrysis galbana*. *Food Chemistry and Toxicology* 68:1144-1148.
- Torres, H. A., Bodey, G. P., Tarrand, J. J. and Kontoyiannis, D. P. (2003) Protothecosis in patients with cancer: case series and literature review. *Clinical Microbiology and Infection* 9:786-792.
- Trookman, N. S., Rizer, R. L. and Weber, T. (2011) Irritation and allergy patch test analysis of topical treatments commonly used in wound care: Evaluation on normal and compromised skin. *Journal of the American Academy of Dermatology* 64:S16-S22.

- Turjanmaa, K., Darsow, U., Niggemann, B., Rance, F., Vanto, T. and Werfel, T. (2006) EAACI/GA²LEN position paper: present status of the atopy patch test*. *Allergy* 61:1377-1384.
- Ueno, R., Hanagata, N., Urano, N. and Suzuki, M. (2005) Molecular phylogeny and phenotypic variation in the heterotrophic green algal genus *Prototheca* (Trebouxiophyceae, Chlorophyta). *Journal of Phycology* 41:1268-1280.
- von Bergen, M., Eidner, A., Schmidt, F., Murugaiyan, J., Wirth, H., Binder, H., Maier, T. and Roesler, U. (2009) Identification of harmless and pathogenic algae of the genus *Prototheca* by MALDI-MS. *Proteomics Clinical Applications* 774-784.
- Wu, H. L., Hseu, R. S. and Lin, L. P. (2001) Identification of *Chlorella* spp. isolates using ribosomal DNA sequences. *Botanical Bulletin of Academia Sinica* 42:115-121.
- Wu, Q. Y. (1994) New discoveries in study on hydrocarbons from thermal degradation of heterotrophically yellowing algae. *Science in China, Series B* 37:326-335.
- Yim, H. E., Yoo, K. H., Seo, W. H., Won, N. H., Hong, Y. S. and Lee, J. W. (2007) Acute tubulointerstitial nephritis following ingestion of *Chlorella* tablets. *Pediatric Nephrology* 22:887-888.

APPENDIX 1

Appendix 1. Food items selected for WAP supplementation with intended levels of use*

Food Product List	Intended use level (ppm)
Milk, soy, ready-to-drink, not baby's	10,000
Milk, imitation, fluid, non-soy, sweetened, flavors other than chocolate	10,000
Yogurt, plain, lowfat milk	13,500
Yogurt, frozen, NS as to flavor, lowfat milk	13,500
Chocolate syrup, reduced fat milk added	13,500
Fruit smoothie drink, made with fruit or fruit juice and dairy products	13,500
Flavored milk drink, whey- and milk-based, flavors other than chocolate	10,000
Instant breakfast, fluid, canned	13,500
Meal supplement or replacement, commercially prepared, ready-to-drink	13,500
Instant breakfast, powder, not reconstituted	13,500
Cream substitute, liquid	10,000
Cream substitute, powdered	10,000
Ice cream, regular, flavors other than chocolate	10,000
Ice cream, regular, chocolate	10,000
Light ice cream, flavors other than chocolate (formerly ice milk)	10,000
Light ice cream, chocolate (formerly ice milk)	10,000
Pudding, chocolate, ready-to-eat, NS as to from dry mix or canned	10,000
Pudding, flavors other than chocolate, ready-to-eat, NS as to from dry mix or canned	10,000
Imitation cheese, American or cheddar type	13,500
Imitation mozzarella cheese	13,500
Salmon cake or patty	10,000
Beef with vegetable (diet frozen meal)	10,000
Chicken and noodles with vegetable, dessert (frozen meal)	10,000
Beef, broth, bouillon, or consommé	10,000
Chicken, broth, bouillon, or consommé	10,000
Gelatin drink, powder, flavored, with low-calorie sweetener, reconstituted	10,000
Gravy, poultry	10,000
Gravy, beef or meat	10,000
Egg substitute, NS as to powdered, frozen, or liquid**	32,000
Scrambled egg, made from powdered mixture**	13,500
Baked beans, vegetarian	10,000
Hummus	13,500
Falafil	10,000
Protein powder, NFS	160,000
Meal replacement or supplement, soy- and milk-base, powder, reconstituted with water	16,000
High protein bar, candy-like, soy and milk base	16,000
Textured vegetable protein, dry	160,000
Meal replacement or supplement, liquid, soy-based	160,000
Tofu, frozen dessert, flavors other than chocolate	13,500
Bacon strip, meatless	13,500
Breakfast link, pattie, or slice, meatless	64,000
Chicken, meatless, NFS	64,000

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Food Product List	Intended use level (ppm)
Fish stick, meatless	13,500
Frankfurter or hot dog, meatless	13,500
Vegetarian burger or patty, meatless, no bun	13,500
Sandwich spread, meat substitute type	10,000
Vegetarian chili (made with meat substitute)	13,500
Tofu and vegetables (including carrots, broccoli, and/or dark-green leafy vegetables (no potatoes)), with soy-based sauce (mixture)	13,500
Vegetarian stew	10,000
Peanut butter	10,000
Biscuit mix, dry	13,500
Bread, pita	13,500
Brioche	10,000
Bagel	13,500
Bread, multigrain	13,500
Bagel, multigrain	13,500
Muffin, English, multigrain	13,500
Muffin, chocolate	13,500
Cake batter, raw, chocolate	13,500
Cake batter, raw, not chocolate	13,500
Cheesecake	13,500
Cake, sponge, without icing	13,500
Cookie, brownie, without icing	13,500
Pie, custard	13,500
Breakfast bar, NFS	13,500
Meal replacement bar	80,000
Snack bar, oatmeal	13,500
Granola bar, oats, fruit and nuts, lowfat	13,500
Granola bar with nuts, chocolate-coated	13,500
Cracker, snack	64,000
Salty snacks, multigrain, chips	64,000
Multigrain mixture, pretzels, cereal and/or crackers, nuts	10,000
Pancakes, plain	10,000
Macaroni, cooked, NS as to fat added in cooking	10,000
Oatmeal, cooked, regular, fat not added in cooking	10,000
Corn flakes, NFS	13,500
Crispy Rice	13,500
Granola, NFS	13,500
100% Bran	13,500
Toasted oat cereal	13,500
Wheat, puffed, plain	13,500
Beef noodle soup	13,500
Chicken noodle soup	13,500
Instant soup, noodle	13,500
Fruit juice blend, 100% juice	13,500
Fruit smoothie drink, made with fruit or fruit juice only (no dairy products)	13,500
Vegetable chips	13,500

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Food Product List	Intended use level (ppm)
Tomato and vegetable juice, mostly tomato	10,000
Spaghetti sauce, meatless	10,000
Mushroom soup, cream of, prepared with milk	64,000
Vegetable soup, prepared with water or ready-to-serve	64,000
Butter, whipped, tub, salted	10,000
Margarine, whipped, tub, salted	10,000
Vegetable oil-butter spread, tub, salted	10,000
Caesar dressing	10,000
French dressing	10,000
Mayonnaise-type salad dressing	10,000
Thousand Island dressing	10,000
Gelatin snacks	10,000
Milk chocolate candy, plain	10,000
Chocolate, sweet or dark	10,000
Chocolate, white	10,000
Rice beverage	13,500
Fruit flavored drink, made from powdered mix	13,500
Fruit-flavored thirst quencher beverage, low calorie	13,500
Fluid replacement, electrolyte solution	13,500
Energy drink	32,000
Fruit-flavored beverage, dry concentrate, with sugar, not reconstituted	13,500

*The food categories correspond to those listed in 21 CFR §170.3(n). ** Fully inspected egg products under FDA jurisdiction. NFS = Not further specified; NS = Not specified; ppm = parts *per* million; WAP = Whole Algal Protein



**DOSSIER IN SUPPORT OF THE GENERALLY RECOGNIZED
AS SAFE (GRAS) STATUS OF WHOLE ALGAL PROTEIN
FROM *CHLORELLA PROTOTHECOIDES* STRAIN S106 AS A
FOOD INGREDIENT**

February 18, 2014

FINAL

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**DOSSIER IN SUPPORT OF THE GENERALLY RECOGNIZED AS SAFE (GRAS)
STATUS OF WHOLE ALGAL PROTEIN FROM *CHLORELLA PROTOTHECOIDES*
STRAIN S106 AS A FOOD INGREDIENT**

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**DOSSIER IN SUPPORT OF THE GENERALLY RECOGNIZED AS SAFE (GRAS)
STATUS OF WHOLE ALGAL PROTEIN FROM *CHLORELLA PROTOTHECOIDES*
S106 AS A FOOD INGREDIENT**

1. EXECUTIVE SUMMARY

Solazyme, Inc. (hereinafter referred to as Solazyme) convened an independent panel of recognized experts (hereinafter referred to as the Expert Panel¹), qualified by their scientific training and relevant national and international experience to evaluate the safety of food ingredients, to determine the Generally Recognized As Safe (GRAS) status of the proposed uses of a whole algal protein (hereinafter referred to as WAP) from *Chlorella protothecoides* Strain S106 as a food ingredient based on scientific procedures. WAP is to be added to foods identified herein, such that the 90th percentile consumption from all categories may be up to 5.56 g/day. Solazyme assures that all relevant, unpublished information in its possession related to the safety of WAP has been supplied to and summarized by the Burdock Group in this dossier. A comprehensive search of the scientific literature was conducted through October 18, 2013 for safety and toxicity information on *Chlorella protothecoides*, WAP and related substances, and has been summarized in this dossier by the Burdock Group. This information, along with supporting documentation, was made available to the Expert Panel. In addition, the Expert Panel independently evaluated additional materials deemed appropriate and necessary. Following an independent, critical evaluation, the Expert Panel conferred and unanimously agreed that the proposed uses of WAP are safe and suitable when consumed up to 5.56 g/day.

2. INTRODUCTION

WAP is a high protein (40 – 75% protein) algal powder composed of the dried biomass of the microalgae *Chlorella protothecoides* Strain S106. WAP will be used as a protein source, analogous to soy- and animal-based proteins, in a variety of foods.² WAP will only be added to foods for which a standard of identity does not exist. This dossier is a summary of the scientific evidence that supports the Generally Recognized As Safe status of WAP as a food ingredient for human consumption.

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.

² Baked goods and mixes, breakfast cereals, meal replacements, cheeses, milk products, dairy and nondairy products, egg products, fish products, meat products, poultry products, plant protein products, grain products and pastas, gravies and sauces, salad dressings, margarines, processed vegetables and vegetable juices, fresh and processed fruit juices, nonalcoholic beverages, gelatins and puddings, frozen dairy, soups, nut products, snack foods and soft candies.

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2.1. Description and Identification of Organism

WAP is a pale yellow to green³ high protein powder composed of the dried biomass of *C. protothecoides* S106. As a species, *C. protothecoides* is currently assigned to the genus *Chlorella* in the phylum Chlorophyta. Widespread in fresh and salt water, soil and air (Wu *et al.*, 2001), the green microalgae *Chlorella spp.* are nonmotile, unicellular eukaryotes, spherical in shape and have diameters that typically range from 2 – 10 µm (Kay, 1991; Becker, 2007). The cell walls are hemicellulosic, rigid, and account for approximately 10% of the algal dry weight (Becker, 2007). *Chlorella spp.* also characteristically have membrane-bound organelles (Kay, 1991). Axenic⁴ cultures of *Chlorella spp.* are easily established, in part because replication under optimum conditions tends to be rapid, often requiring less than two hours (Kay, 1991). Protein and lipid content and the fatty acid profile are known to vary widely with the species, the stage of cell growth (exponential or stationary), and with environmental conditions (*e.g.*, available nutrients, temperature, and light intensity) (Kay, 1991). In previous reports, the protein content of *Chlorella spp.* have, for example, measured from 15 – 60 % protein on a dry weight basis (Kay, 1991; Becker, 1994).

Although the strains are all unicellular and morphologically similar, the species grouped into the genus *Chlorella* have recently been recognized to be phylogenetically divergent (Ferris *et al.*, 2005) and not necessarily related (Tiberg and Einarsson, 1989; Huss *et al.*, 1999). Nomenclature and taxonomic assignments based on traditional identification methods are being re-examined – and when necessary, reclassified – using data from genetic sequence analysis (Ferris *et al.*, 2005). Because species comprising the *Chlorella* genus are a taxonomically complicated group, chemotaxonomic character, DNA⁵ base composition and DNA/DNA hybridization are all used to distinguish the morphologically similar species from one another (Huss *et al.*, 1999; Ferris *et al.*, 2005). For example, based on comparative analyses of 18S ribosomal RNA gene sequences, Huss *et al.* (1999) have recommended that only four species remain in the *Chlorella* genus: *C. vulgaris*, *C. lobophora*, *C. sorokiniana*, and *C. kessleri*.

Solazyme obtained *C. protothecoides* UTEX 250 from the University of Texas (UTEX) Culture Collection of Algae in Austin, Texas and assigned the strain the Solazyme internal strain number ‘S106’. UTEX 250 had, in turn, been obtained by UTEX as *C. protothecoides* CCAP211/7C from the Culture Collection of Algae and Protozoa (CCAP), Scottish Association for Marine Science, Argyll Scotland, United Kingdom, where the strain was originally deposited sometime between 1952 and 1955 by A.J. Kluyver⁶ as the third isolate of *C. vulgaris* (‘3 *C. vulgaris*’) taken from freshwater in Delft, Netherlands. Other deposits of this strain include the Meyer 34 and SAG211-7C accessions in the Sammlung von Algenkulturen Göttingen (SAG,

³ WAP is not added to food with the intention of acting as a color. Although WAP (which is naturally light yellow to green) can modify the color of pale foods in the same way that adding chocolate or tomatoes can alter color, the ingredient will be used in such a way that any color imparted is clearly unimportant insofar as the appearance, value, marketability or consumer acceptability is concerned. WAP is, therefore, exempt from the definition of a color additive and from FDA premarket approval requirements for color additives [FFDCA §201(t) and 21 CFR§70.3(g)].

⁴ Cultures containing a single strain (*i.e.*, not contaminated by or associated with other microorganisms)

⁵ DNA = deoxyribonucleic acid

⁶ <<http://web.biosci.utexas.edu/utex/algaeDetail.aspx?algaeID=2779>>; site accessed October 11, 2013.

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Albrecht-von-Haller-Institute for Plant Science, University of Göttingen, Göttingen, Germany).⁷ Additional designations by which this strain is known include *C. protothecoides* var. *communis* Shihira and Krauss (1965),⁸ *C. protothecoides* Krüger and *Auxenochlorella protothecoides* (Krüger) Kalina & Puncochárová (1987). Based on accepted designations, UTEX 250 (S106) may be appropriately considered a strain of *C. protothecoides* or *Auxenochlorella protothecoides*. All *C. protothecoides* (*A. protothecoides*) strains held at the American Type Culture Collection (ATCC, Rockville, Maryland) are preserved under Biosafety Level (BSL) 1 conditions which indicate the microorganisms are not recognized to cause disease in immunocompetent adult humans.⁹

The ecophysiological and biochemical characteristics of *C. protothecoides* include the inability to utilize nitrate as a substrate, the need for thiamine but not for vitamin B₁₂, the lack of secondary carotenoids (most strains), and a tolerance for high temperatures (28 – 34 °C upper limits), acidic conditions (3.5 – 4.0 pH) and high salt concentrations (3 – 4%) (Huss *et al.*, 2002). Morphology for the species includes the absence of pyrenoids, but the presence of a tri-laminar layer within the cell wall (Huss *et al.*, 2002). Reproduction is by endosporulation, an asexual process through which two to twelve sporangiospores are produced and released by each parental sporangium during vegetative growth (Ramírez-Romero *et al.*, 2010). When glucose is available as a carbon source and inorganic nitrogen is not restricted, the cells produce high levels of protein (Day *et al.*, 2009).

Although similar in appearance, morphology, and composition to other *Chlorella* species, such as *C. vulgaris* and *C. pyrenoidosa*¹⁰ (Robinson and Guzman-Juarez, 1978; Kay, 1991; Brown and Jeffery, 1992; Tokusoglu and Unal, 2003; Ravishankar, *et al.*, 2006; Day *et al.*, 2009) *C. protothecoides* alone has the ability, when exposed to glucose, to ‘etiolize’ (*i.e.*, ‘de-green’). Genetically, *C. protothecoides* strains (with the single exception of *C. protothecoides* var. *acidicola*¹¹) have been shown to be closely related to species in the achlorophyllous genus *Prototheca*. Gene-based phylogenetic trees developed from nuclear 18S rRNA,¹² plastid 16S rRNA, SSU¹³ and LSU¹⁴ rDNA,¹⁵ and 26S rDNA domain sequences have repeatedly demonstrated that *A. protothecoides* UTEX 25 (S485), the type strain for *A. protothecoides*, is closely related to the *Prototheca* species (Huss *et al.*, 1999; Tartar *et al.*, 2003; Ueno *et al.*, 2005; Satoh *et al.*, 2010). At least one protein-based phylogenetic tree also confirms these assessments

⁷ <http://sagdb.uni-goettingen.de/detailedList.php?str_number=211-7c>; site accessed October 11, 2013.

⁸ <<http://web.biosci.utexas.edu/utex/algaeDetail.aspx?algaeID=2779>>; site accessed October 11, 2013.

⁹ <http://www.atcc.org/Search_Results.aspx?dsNav=Ntk:PrimarySearch%7cchlorella+protothecoides%7c3%7c,Ny:True,Ro:0,N:1000552&searchTerms=chlorella+protothecoides&redir=1>; site accessed October 11, 2013.

¹⁰ Many of these stains have been reclassified as *C. sorokiniana* or *C. fusca* var. *vacuolata*.

¹¹ Analysis of 18S RNA gene sequences has demonstrated only a weak phylogenetic relationship to *C. protothecoides* var. *acidicola* which is no longer considered a variety of *C. protothecoides* (Huss *et al.*, 2002; Ferris *et al.*, 2005).

¹² rRNA = ribosomal ribonucleic acid

¹³ SSU = small subunit of ribulose bis-phosphate carboxylase-oxygenase

¹⁴ LSU = large subunit of ribulose bis-phosphate carboxylase-oxygenase

¹⁵ rDNA = ribosomal deoxyribonucleic acid

(von Bergen *et al.*, 2009). The relationship is sufficiently homologous that comparative analysis of the phylogeny of the related *A. protothecoides* UTEX 25 (S485) and twelve strains of *Prototheca* were found to form a monophyletic clade (Ueno *et al.*, 2005).

2.1.1. Corroborative Genotypic Information

As discussed above, delineation of microalgal species based on morphology is problematic due to potentially strong morphological and physiological similarities among genetically diverse species. Molecular indicators such as plastidic 23S rDNA sequence homology are more reliable indicators of strain relatedness. As an example, the plastid 23S rDNA sequences shown in Figure 1 demonstrate that *C. protothecoides* S106 (UTEX 250 (Solazyme source); also known as (aka) SAG 211-7C and CCAP 211/7C; original deposition CCAP (1952-5)) possesses a genotype identical (*i.e.*, having 100% homology) to that of Solazyme designated strain *C. protothecoides* S485 (UTEX 25 (Solazyme source); aka, SAG 211-7A, CCAP211/7A, and ATCC 30407) (Solazyme Inc., 2011). Strain S485 is known formally as *Auxenochlorella protothecoides* (Krüger) Kalina & Puncochárová (1987) and is the type strain for *A. protothecoides*. *C. protothecoides* S106 is also shown to share approximately 87.5% homology with another *C. protothecoides* strain, S102 (UTEX B 25 (Solazyme source); aka original deposition CCAP (1952-5)) which is called *C. protothecoides* Krüger (1892) and *A. protothecoides*. In summary, based on plastid 23S rDNA sequencing, *C. protothecoides* S106 is identical (*i.e.* 100% homology) to *A. protothecoides* S485 (type strain of *Auxenochlorella protothecoides*) and only somewhat closely related (approximately 87.5% homology) to *C. protothecoides* S102.

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S102 23S TGTTGAAGAATGAGCCGGCGACTTAGAAAACGTGGCAAGGTTAAGGAAACGTATCCGGAG
 S106 23S TGTTGAAGAATGAGCCGGCGACTTAGAAAAAGTGGCGTGGTTAAGGAAAAAT-TCCGAAG
 S485 23S TGTTGAAGAATGAGCCGGCGACTTAGAAAAAGTGGCGTGGTTAAGGAAAAAT-TCCGAAG

 S102 23S CCGAAGCGAAAGCAAGTCTGAACAGGGCG-----ATTAAGTCA
 S106 23S CCTTAGCGAAAGCGAGTCTGAATAGGGCGATCAAATATTTAATATTTACAATTTAGTCA
 S485 23S CCTTAGCGAAAGCGAGTCTGAATAGGGCGATCAAATATTTAATATTTACAATTTAGTCA

 S102 23S TTTTTTCTAGACCCGAACCCGGGTGATCTAACCATGACCAGGATGAAGCTTGGGTGACAC
 S106 23S TTTTTTCTAGACCCGAACCCGGGTGATCTAACCATGACCAGGATGAAACTTGGGTGATAC
 S485 23S TTTTTTCTAGACCCGAACCCGGGTGATCTAACCATGACCAGGATGAAACTTGGGTGATAC

 S102 23S CAAGTGAAGGTCCGAACCGACCGATGTTGAAAAATCGGCGGATGAGTTGTGGTTAGCGGT
 S106 23S CAAGTGAAGGTCCGAACCGACCGATGTTGAAAAATCGGCGGATGAGTTGTGGTTAGCGGT
 S485 23S CAAGTGAAGGTCCGAACCGACCGATGTTGAAAAATCGGCGGATGAGTTGTGGTTAGCGGT

 S102 23S GAAATACCAGTCGAACTCGGAGCTAGCTGGTCTCCCCGAAATGCGTTGAGGCGCAGCGG
 S106 23S GAAATACCAGTCGAAACCCGGAGCTAGCTGGTCTCCCCGAAATGCGTTGAGGCGCAGCAG
 S485 23S GAAATACCAGTCGAAACCCGGAGCTAGCTGGTCTCCCCGAAATGCGTTGAGGCGCAGCAG

 S102 23S TTCATA-AGGCTGTCTAGGGGTAAAGCACTGTTTCGGTGCGGGCTGCGAAAACGGTACCA
 S106 23S TACATCTAGTCTATCTAGGGGTAAAGCACTGTTTCGGTGCGGGCTGTGAAAACGGTACCA
 S485 23S TACATCTAGTCTATCTAGGGGTAAAGCACTGTTTCGGTGCGGGCTGTGAAAACGGTACCA

 S102 23S AATCGTGGCAAACCTCTGAATACTAGATATG-CTATTTATGGGCCAGTGAGACGGTGGGGG
 S106 23S AATCGTGGCAAACCTCTGAATACTAGAAATGACGGTGTA-GT---AGTGAGACTGTGGGGG
 S485 23S AATCGTGGCAAACCTCTGAATACTAGAAATGACGGTGTA-GT---AGTGAGACTGTGGGGG

 S102 23S ATAAGCTTCATCGTCGAGAGGGAAACAGCCCAGATCACTAGCTAAGGCCCAAAATGATC
 S106 23S ATAAGCTCCATTGTCAAGAGGGAAACAGCCCAGACCACCAGCTAAGGCCCAAAATGGTA
 S485 23S ATAAGCTCCATTGTCAAGAGGGAAACAGCCCAGACCACCAGCTAAGGCCCAAAATGGTA

 S102 23S GTTAAAGTGACAAAGGAGGTGAGAATGCAGAAACAACCAGGAGGTTTGCTTAGAAGCAGCC
 S106 23S ATGTAGTGACAAAGGAGGTGAAAATGCAACACAACCAGGAGGTTGGCTTAGAAGCAGCC
 S485 23S ATGTAGTGACAAAGGAGGTGAAAATGCAACACAACCAGGAGGTTGGCTTAGAAGCAGCC

 S102 23S ACCCTTTAAAGAGTGCGTAATAGCTCACTG
 S106 23S ATCCTTTAAAGAGTGCGTAATAGCTCACTG
 S485 23S ATCCTTTAAAGAGTGCGTAATAGCTCACTG

Figure 1. Plastid 23S rDNA sequences for Solazyme designated *C. protothecoides* strains S102, S106 (Solazyme strain of interest) and S485 (*Auxenochlorella protothecoides* type strain)

In similar fashion, Solazyme's *C. protothecoides* S106 strain was compared using partial plastidic 23S rDNA sequencing with 21 *C. vulgaris* strains obtained from five different depository collections (Solazyme Inc., 2011). As indicated below, each test strain is identified by its Solazyme designation, common name and strain source; identical strains and other current or previous names are included as parenthetical information for ease of reference:

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- (1) S183: *C. vulgaris*, UTEX 395 (formerly *C. pyrenoidosa* (Starr and Zeikus 1987));
- (2) S184: *C. vulgaris* (type strain), UTEX 259, (CCAP 211/11B; aka *Chlorella candida*; *Chlorella miniata*; *Chlorella vulgaris* var. *vulgaris* f. *vulgaris*; original deposition CCAP 1952-5);
- (3) S185: *C. vulgaris*, UTEX 1809 (formerly *C. salina* (Starr and Zeikus 1987));
- (4) S187: *C. vulgaris*, UTEX 2714 (*Chlorella vulgaris* Bashan);
- (5) S188: *C. vulgaris*, UTEX 30 (CCAP 211/12; SAG 211-12; ATCC 16487; aka *C. vulgaris* Beijerinck f. *viridis* ((Chodat) Fott and Nováková));
- (6) S190: *C. vulgaris*, UTEX 396 (formerly *C. vulgaris* var. *viridis* (Starr and Zeikus 1987));
- (7) S191: *C. vulgaris*, UTEX 26 (formerly *Chlorella pyrenoidosa* (Starr & Zeikus 1987); aka *C. photophila* (Shihira & Krauss 1965); *C. emersonii*; *C. fusca* var. *vacuolata*);
- (8) S192: *C. vulgaris*, UTEX 265 (CCAP 211/11J; SAG 211-11J; *Chlorella vulgaris* Beijerinck 1890 (Rodhe); aka *C. simplex* (Shihira & Krauss 1965) and *Chlorella vulgaris* var. *vulgaris* f. *vulgaris*; original designation CCAP 1630);
- (9) S246: *C. vulgaris*, SAG 30.80 (*Chlorella vulgaris* Beijerinck (Senger 1965); formerly *C. saccharophila*);
- (10) S257: *C. vulgaris*, SAG 211.11T (*Chlorella vulgaris* Beijerinck (Ruschmann));
- (11) S300: *C. vulgaris*, CCAP 211/19 (*Chlorella vulgaris* Beijerinck 1890 (von Witsch 1946/7));
- (12) S301: *C. vulgaris*, CCAP 211/11S (*Chlorella vulgaris* Beijerinck 1890 (Pirson));
- (13) S302: *C. vulgaris*, CCAP 211/11Q (*Chlorella vulgaris* Beijerinck (Czurda));
- (14) S303: *C. vulgaris*, CCAP 211/11P (SAG 211-11P; *Chlorella vulgaris* Beijerinck (Algéus 1942 Strain B);
- (15) S344: *C. vulgaris*, CCAP 211/81 (*Chlorella vulgaris* Beijerinck 1890 (Krienitz 1979); original designation CCAP A36);
- (16) S345: *C. vulgaris*, CCAP 211/80 (*Chlorella vulgaris* Beijerinck 1890 (Krienitz 1979); original designation CCAP A35);
- (17) S410: *C. vulgaris*, CCALA¹⁶ 263 (*C. vulgaris* Beijerinck (Hindak 1967/61));
- (18) S412: *C. vulgaris*, CCALA 266 (*C. vulgaris* Beijerinck (Marsalek 1985/57));

¹⁶ Culture Collection of Autotrophic Organisms (CCALA), Institute of Botany, Academy of Sciences of the Czech Republic, Třeboň, Czech Republic; <<http://www.butbn.cas.cz/ccala/index.php?page=sr&cbl=Algae>>; site accessed October 11, 2013.

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- (19) S413: *C. vulgaris*, CCALA 268 (*C. vulgaris* Beijerinck (Gaffron/Bethesda C 1.3.1.));
(20) S497: *C. vulgaris*, CAUP¹⁷ H1993 (*C. vulgaris* Beijerinck (Punčochářová 1981/22)); and
(21) S498: *C. vulgaris*, CAUP H1996 (*C. vulgaris* Beijerinck f. *globosa* (Andreeva 1961); *C. vulgaris* var. *vulgaris* f. *globosa*).

Distance values (D values), a numerical metric used to indicate genetic relatedness, were generated from the sequence data (Solazyme Inc., 2011); lower D-values indicate sequences that are more closely related with '0.000' indicating identical sequences (*i.e.*, 100% homology). Higher values, on the other hand, indicate more divergent sequences. Isolates with minor genetic variation (high homology) are generally accepted as varying by a value of only about 0.005% (Rawat, *et al.*, 2005). For perspective, strains having less than 95.5% homology (based on 16S rRNA sequence identity) are routinely regarded as different species (Stackebrandt and Goebel, 1994). Based on an evaluation of the numeric metric used to assess relatedness (Table 1), the partial plastidic 23S rDNA sequence for Solazyme strain S106, the *C. protothecoides* strain of interest, had D values ≥ 0.080 relative to the 21 *C. vulgaris* strains examined. When the D values for Solazyme strain S184 (the type strain for *C. vulgaris*) are considered, S184 is seen to be (1) identical to 15 of the other 21 *C. vulgaris* strains tested (D value = 0.000 for all),¹⁸ (2) very closely or reasonably closely matched to three strains (D values from 0.002 to 0.034),¹⁹ and (3) more distantly related to the remaining three strains (D values ≥ 0.090).²⁰ S106 is counted among this last group, that is, it is more distantly related to the type strain for *C. vulgaris*. An additional comparison of the 23S sequence of *C. protothecoides* S106 using nBLAST analysis to that of GenBank accession No.²¹ L43357 *C. vulgaris* Beijerinck (NIES²²-2170, formerly in the IAM²³ collection as C-27; formerly classified as *C. ellipsoidea* Gerneck) determines homology of 87% (FDA, 2012). In summary, *C. protothecoides* S106 is distantly related to many of the *C. vulgaris* strains tested, including S184 (UTEX 259), but it is also notable that the *C. vulgaris* type strain S184 is more closely related to S106 than to some *C. vulgaris* isolates (*e.g.*, S412 and S498).

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¹⁷ Culture Collection of Algae of Charles University in Prague (CAUP), Praha, Czech Republic; <<http://botany.natur.cuni.cz/algo/caup-list.htm>>; site accessed October 11, 2013.

¹⁸ S183, S185, S188, S190, S192, S246, S257, S300, S302, S303, S344, S345, S410, S413, and S497

¹⁹ S187, S197, and S301

²⁰ S106, S412, and S498

²¹ No. = number

²² National Institute for Environmental Studies, Tsukuba, Japan.

²³ Institute of Applied Microbiology, Center for Cellular and Molecular Research, Institute of Molecular and Cellular Biosciences University of Tokyo, Tokyo, Japan.

Table 1. Tabulated Distance values for 23S rDNA sequences of *C. protothecoides* S106 (Solazyme strain of interest) compared to S183, S184 (*C. vulgaris* type strain), S185, S187, S188, S190, S191, S192, S246, S257, S300, S301, S302, S303, S344, S345, S410, S412, S413, S497, and S498. (Lower values indicate sequences that are more closely related, with '0.000' indicating identical sequences. Higher values indicate more divergent sequences.)

	S106 23S	S183 23S	S184 23S	S185 23S	S187 23S	S188 23S	S190 23S	S191 23S	S192 23S	S246 23S	S257 23S	S300 23S	S301 23S	S302 23S	S303 23S	S344 23S	S345 23S	S410 23S	S412 23S	S413 23S	S497 23S	S498 23S		
S106 23S		0.291	0.050	0.291	0.050	0.291	0.050	0.291	0.050	0.291	0.050	0.291	0.050	0.291	0.052	0.291	0.052	0.291	0.053	0.291	0.053	0.291	0.053	0.291
S183 23S	0.090		0.000	0.200	0.050	0.200	0.050	0.200	0.050	0.200	0.050	0.200	0.050	0.200	0.054	0.200	0.050	0.200	0.050	0.200	0.050	0.200	0.050	0.200
S184 23S	0.090	0.200		0.200	0.050	0.200	0.050	0.200	0.050	0.200	0.050	0.200	0.050	0.200	0.054	0.200	0.050	0.200	0.050	0.200	0.050	0.200	0.050	0.200
S185 23S	0.090	0.200	0.000		0.050	0.200	0.050	0.200	0.050	0.200	0.050	0.200	0.050	0.200	0.054	0.200	0.050	0.200	0.050	0.200	0.050	0.200	0.050	0.200
S187 23S	0.090	0.291	0.050	0.291		0.291	0.050	0.291	0.050	0.291	0.050	0.291	0.050	0.291	0.056	0.291	0.053	0.291	0.053	0.291	0.053	0.291	0.053	0.291
S188 23S	0.090	0.200	0.050	0.200		0.200	0.050	0.200	0.050	0.200	0.050	0.200	0.050	0.200	0.054	0.200	0.050	0.200	0.050	0.200	0.050	0.200	0.050	0.200
S190 23S	0.090	0.200	0.050	0.200	0.050		0.200	0.050	0.200	0.050	0.200	0.050	0.200	0.050	0.054	0.200	0.050	0.200	0.050	0.200	0.050	0.200	0.050	0.200
S191 23S	0.095	0.202	0.052	0.202	0.052	0.202		0.202	0.052	0.202	0.052	0.202	0.052	0.202	0.056	0.202	0.052	0.202	0.052	0.202	0.052	0.202	0.052	0.202
S192 23S	0.090	0.200	0.050	0.200	0.050	0.200	0.050		0.200	0.050	0.200	0.050	0.200	0.054	0.200	0.050	0.200	0.050	0.200	0.050	0.200	0.050	0.200	0.200
S246 23S	0.090	0.200	0.050	0.200	0.050	0.200	0.050	0.200		0.200	0.050	0.200	0.050	0.200	0.054	0.200	0.050	0.200	0.050	0.200	0.050	0.200	0.050	0.200
S257 23S	0.090	0.200	0.050	0.200	0.050	0.200	0.050	0.200	0.200		0.200	0.054	0.200	0.050	0.200	0.050	0.200	0.050	0.200	0.050	0.200	0.050	0.200	0.200
S300 23S	0.090	0.200	0.050	0.200	0.050	0.200	0.050	0.200	0.200	0.200		0.054	0.200	0.050	0.200	0.050	0.200	0.050	0.200	0.050	0.200	0.050	0.200	0.200
S301 23S	0.092	0.294	0.054	0.294	0.054	0.294	0.054	0.294	0.054	0.294	0.054	0.294		0.294	0.054	0.294	0.054	0.294	0.054	0.294	0.054	0.294	0.054	0.294
S302 23S	0.090	0.200	0.050	0.200	0.050	0.200	0.050	0.200	0.200	0.200	0.200	0.054	0.200		0.050	0.200	0.050	0.200	0.050	0.200	0.050	0.200	0.050	0.200
S303 23S	0.092	0.200	0.050	0.200	0.051	0.200	0.050	0.200	0.200	0.200	0.200	0.054	0.200	0.200		0.200	0.050	0.200	0.050	0.200	0.050	0.200	0.050	0.200
S344 23S	0.090	0.200	0.050	0.200	0.050	0.200	0.050	0.200	0.200	0.200	0.200	0.054	0.200	0.050	0.200		0.050	0.200	0.050	0.200	0.050	0.200	0.050	0.200
S345 23S	0.090	0.200	0.050	0.200	0.050	0.200	0.050	0.200	0.200	0.200	0.200	0.054	0.200	0.050	0.200	0.200		0.200	0.050	0.200	0.050	0.200	0.050	0.200
S410 23S	0.090	0.200	0.050	0.200	0.050	0.200	0.050	0.200	0.200	0.200	0.200	0.054	0.200	0.050	0.200	0.050	0.200		0.050	0.200	0.050	0.200	0.050	0.200
S412 23S	0.150	0.101	0.176	0.101	0.110	0.101	0.176	0.101	0.176	0.101	0.176	0.101	0.176	0.101	0.112	0.101	0.103	0.101	0.103	0.101	0.103	0.101	0.103	0.101
S413 23S	0.090	0.200	0.050	0.200	0.050	0.200	0.050	0.200	0.200	0.200	0.200	0.054	0.200	0.050	0.200	0.050	0.200	0.050	0.200	0.050	0.200	0.050	0.200	0.200
S497 23S	0.090	0.200	0.050	0.200	0.050	0.200	0.050	0.200	0.200	0.200	0.200	0.054	0.200	0.050	0.200	0.050	0.200	0.050	0.200	0.050	0.200	0.050	0.200	0.200
S498 23S	0.136	0.151	0.122	0.151	0.148	0.151	0.122	0.151	0.122	0.151	0.122	0.151	0.122	0.151	0.150	0.151	0.151	0.151	0.151	0.151	0.151	0.151	0.151	0.151

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Solazyme's *C. protothecoides* S106 strain was also compared to eight strains in Solazyme's collection that had previously (prior to 1992) been classified as *C. pyrenoidosa*. As *C. pyrenoidosa*, these strains had been the subject of several published nutritional studies. With the advent of modern molecular methods of identification, the species in the *Chlorella* genus have undergone significant taxonomic revision. In 1992, many *C. pyrenoidosa* strains were re-evaluated and reclassified as *C. sorokiniana* (Kessler and Huss, 1992) or *C. fusca* var. *vacuolata*. As indicated below, each test strain is identified by Solazyme designation, current classification and strain source; previous names and identical strains and/or other current or former names are also included as parenthetical information:

- (1) S129: *C. sorokiniana*, UTEX 1230 as *C. pyrenoidosa* (formerly *C. pyrenoidosa* (Starr & Zeikus 1987); *C. vulgaris* fo. *tertia* and *C. vulgaris* group; ATCC 22521; CCAP 211/8K as *C. vulgaris* f. *tertia*; CCAO 259 as *C. sorokiniana*; SAG 211-8K as *C. sorokiniana*; originally designated Tx 7-11-05);
- (2) S134: *C. sorokiniana* UTEX 1666, (formerly *C. pyrenoidosa*);
- (3) S135: *C. sorokiniana* UTEX 1670 (formerly *C. pyrenoidosa* (Starr & Zeikus 1987); originally designated B1E-B2P);
- (4) S140: *C. sorokiniana* UTEX B1810 (formerly *C. sorokiniana* var. *pacificensis* (Starr & Zeikus 1987));
- (5) S167: *C. fusca* var. *vacuolata* UTEX 251 (formerly *C. pyrenoidosa*; SAG 211-8B; CCAP 211/8B; CCAUP H 6401; ATCC 11469);
- (6) S168: *C. fusca* var. *vacuolata* UTEX 252 (formerly *C. pyrenoidosa*; aka *Scenedesmus* sp.; *Chlorella emersonii* var. *emersonii*; SAG 211-8C as *C. fusca*; CCAP 211/8C as *Chlorella emersonii* var. *globosa* (Shihira & Kraus 1965));
- (7) S545: *C. sorokiniana* UTEX 1664; and
- (8) S547: *C. sorokiniana* UTEX 1668 (formally *C. pyrenoidosa* (Starr and Zeikus 1987)).

Evaluation of the D values generated from partial plastidic 23S rDNA sequencing (Table 2) indicates that Solazyme strain S106, the *C. protothecoides* strain of interest, has D values ≥ 0.076 ($\leq 88\%$ homology), which shows slightly greater homology to five of the strains previously classified as *C. pyrenoidosa* than was seen between S106 and the 21 previously examined *C. vulgaris* isolates. In addition, Solazyme strains S129, S134 (UTEX 1666), S135 (UTEX 1670), S545 (UTEX 1664), and S547 (UTEX 1668) are seen to be identical (D value = 0.000 for all), which is not surprising, considering that UTEX strains 1664 through 1671 are mutants of UTEX 1663, a sub-isolate of Tx-7-11-05 (UTEX 1230 or S129). As with the *C. vulgaris* isolates examined above, strain S106 is less closely related to most of the strains previously classified as *C. pyrenoidosa* than they are to one another. The exception is strain S168, whose 23S sequence shows less homology to the other strains in this set than S106 shows to the other strains.

In summary, partial, plastidic 23S rDNA sequences were used to assess the genetic relatedness between S106, *C. protothecoides* and two genera of *Chlorella* previously studied and used as foods or dietary supplements, namely *C. vulgaris* and *C. pyrenoidosa*. Algal strain S106

is less closely related to these isolates than to S485 (type strain for *A. protothecoides*), against which S106 demonstrated 100% homology.

Table 2. Tabulated Distance values for 23S rDNA sequences of *C. protothecoides* S106 (Solazyme strain of interest) compared to other *Chlorella* strains S129, S134, S135, S140, S167, S168, S545, and S547. (Lower values indicate sequences that are more closely related, with '0.000' indicating identical sequences. Higher values indicate more divergent sequences.)

	S106 23S	S129 23S	S134 23S	S135 23S	S140 23S	S167 23S	S168 23S	S545 23S	S547 23S
S106 23S		0.076	0.076	0.076	0.086	0.090	0.182	0.076	0.076
S129 23S	0.076		0.000	0.000	0.022	0.049	0.158	0.000	0.000
S134 23S	0.076	0.000		0.000	0.022	0.049	0.158	0.000	0.000
S135 23S	0.076	0.000	0.000		0.022	0.049	0.158	0.000	0.000
S140 23S	0.086	0.022	0.022	0.022		0.041	0.164	0.022	0.022
S167 23S	0.090	0.049	0.049	0.049	0.041		0.142	0.049	0.049
S168 23S	0.182	0.158	0.158	0.158	0.164	0.142		0.158	0.158
S545 23S	0.076	0.000	0.000	0.000	0.022	0.049	0.158		0.000
S547 23S	0.076	0.000	0.000	0.000	0.022	0.049	0.158	0.000	

2.1.2. History of use

Microalgae, such as *Chlorella* spp., have been an accepted part of the human diet for centuries, especially among the populations of Asia and sub-Saharan Africa (Kay, 1991; Ravishankar, *et al.*, 2006). Currently, wild stocks of various microalgae are harvested as food sources not only in Asia (*e.g.*, China, Japan, Burma, Thailand, India, Mongolia, and Siberia), but also in Central and South America (*e.g.*, Mexico, Bolivia, Ecuador, and Peru), Pacific island nations such as Fiji, and Hawaii in the U.S. (Kay, 1991). Of the microalgae that are commercially cultured, those most popular in the U.S. include, but are not limited to, the green microalgae *Chlorella* spp. and *Scenedesmus* spp., and the cyanobacteria *Spirulina* spp. and *Aphanizomenon flos-aquae* spp. (Kay, 1991). In the early 1970s, U.S. companies cultivating microalgae for human and animal use included Earthrise Farms²⁴ in California, Cell Tech²⁵ in Oregon and Cyanotech Corporation²⁶ in Hawaii (originally, in Washington state) (Kay, 1991). Internationally, Sun Chlorella²⁷ in Japan and SOSA Texcoco/Spirulina Mexicana²⁸ in Mexico also grew microalgae during this same period.

²⁴ <<http://www.earthrise.com/>>; site accessed October 11, 2013.

²⁵ Currently SimpleXity Health, Inc. (<<http://www.simplexityhealth.com/>>; site accessed October 11, 2013).

²⁶ <<http://www.cyanotech.com/>>; site accessed October 11, 2013.

²⁷ <<http://www.sunchlorella.com/>>; site accessed October 11, 2013.

²⁸ The world's first large plant for processing microalgae (*Spirulina* spp.) was built on Lake Texacoco in the 1970s. Although Mexican *Spirulina* was first imported into the U.S. for use in health food products in 1979, in 1982 importation was blocked by U.S. authorities due to quality concerns related to pollution of the lake system. In addition to health food products, *Spirulina* was also used in animal and aquaculture feeds. *Spirulina Mexicana* has been closed for several years (<<http://www.spirulinasource.com/>>; site accessed October 11, 2013).

Modern interest in *Chlorella* as a protein source for humans and animals and consumption of various *Chlorella* preparations began 50 to 60 years ago when large-scale culture production became technically feasible, albeit not often cost effective. Early manufacturing facilities were constructed in the U.S. in 1951 (first pilot plant) and in Taiwan in the 1960s (Ravishankar, *et al.*, 2006). Unexpected technical challenges and high production costs (Robinson and Guzman-Juarez, 1978; Graziani *et al.*, 2013) prevented the cultivation of microalgae such as *Chlorella* from developing beyond niche markets such as health food, cosmetics, and animal food (Nasseri *et al.*, 2011; Skjånes *et al.*, 2013). Recent technological advances in the controlled fermentation and recovery processes needed to produce microalgal-derived food ingredients, including those from photosynthetic eukaryotes such as *Chlorella* (Draaisma *et al.*, 2013; Graziani *et al.*, 2013) have made this production economically feasible. Combined with the current global emphasis on developing sustainable food sources, these factors have encouraged a renewed interest in microalgal sources of dietary protein (Draaisma *et al.*, 2013). In 2004, annual global production of microalgae (all species) was estimated to have reached 10,000 tons (Becker, 2007).

Due to the large amounts of protein that *Chlorella* naturally generates (~60% protein) (Ravishankar, *et al.*, 2006; Draaisma *et al.*, 2013), the microalga has been described as a 'nutritional powerhouse' (Ravishankar, *et al.*, 2006). Although *Chlorella* as a food or ingredient in food initially promised versatile usage, its distinctive odor and taste has proven to be a limiting factor due to unpalatability at higher inclusion rates (Powell and Nevels, 1961; Dam *et al.*, 1965; Durrant and Jolly, 1969; Becker, 2007; Skjånes *et al.*, 2013). An additional concern regarding food applications has been the cellulosic cell wall which is not readily digested (Powell and Nevels, 1961; Dam *et al.*, 1965; Durrant and Jolly, 1969; Robinson and Guzman-Juarez, 1978); to ensure access to proteins and other nutrients inside, the cells are usually broken mechanically during production (Kay, 1991; Becker, 2007; Christaki *et al.*, 2011; Nasseri *et al.*, 2011; Skjånes *et al.*, 2013). Currently, the major consuming populations of *Chlorella* and *Chlorella*-type foods are found in China, Korea, and Japan (Day *et al.*, 2009). In U.S. markets, consumption of *Chlorella* biomass is primarily in the form of dietary supplements which are widely available (Day *et al.*, 2009). At least twelve different brands of *Chlorella* products are available in forms that include powder, tablet and capsule with recommended daily intakes ranging from 200 mg/day up to 10 g/day (Hendler and D. Rorvik, 2008; FDA, 2012). National retailers, such as Vitamin Shoppe²⁹ and GNC,³⁰ both carry *Chlorella* products. Among the many strains in the *Chlorella* genus, those that have been most commonly used in foods and dietary supplements belong to the *C. vulgaris*, *C. pyrenoidosa*, *C. fusca*³¹, *C. kessleri*,³¹ and *C. sorokiniana*³² species. Although the scientific literature and electronic searches have not revealed any documented historical usage of high-protein *C. protothecoides* (or *A. protothecoides*) materials, the FDA has responded with "no questions" for GRAS notifications for *C.*

²⁹ <<http://www.vitaminshoppe.com>>; site accessed October 11, 2013.

³⁰ <<http://www.gnc.com>>; site accessed October 11, 2013.

³¹ Prior to the advent of gene-based identification methods, most strains currently designated as *C. fusca*, *C. kessleri*, and *C. sorokiniana* were at one point assigned to *C. pyrenoidosa*.

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protothecoides algal oil (FDA, 2012) and *C. protothecoides* S106 flour with 40-70% lipid (FDA, 2013) for use in human food.

2.1.3. Current uses

As they are economically promising sources of lutein (xanthophyll), *C. protothecoides* strains such as UTEX 29 are being investigated as potential replacements for French marigold (*Tagetes patula*), the petals of which are currently harvested and extracted for the carotenoid antioxidant (Wei *et al.*, 2008; Fernández-Sevilla *et al.*, 2010). Lutein, a natural constituent of many foods (e.g., oranges, lettuce, broccoli, spinach, eggs) is also included in feed as an indirect colorant for animal tissues and is frequently included as an ingredient in foods, dietary supplements, and cosmetics, and as a component in drug products (Wei *et al.*, 2008; Fernández-Sevilla *et al.*, 2010).

C. protothecoides including UTEX 25, the *A. protothecoides* type strain, are currently being developed for large scale cultivation of high quality³² biodiesel feedstock (Xiong *et al.*, 2008; Xiong *et al.*, 2010; Sirisansaneeyakul *et al.*, 2011).

C. protothecoides is used to assess the toxicity of effluent from bleached kraft pulp mills by the National Pulp Mills Research Program³³ in Australia (Neil *et al.*, 2009) and is one of three aquatic organisms (the other two being the water flea *Ceriodaphnia cf. dubia* and the ciliated protozoan *Tetrahymena thermophila*) used to monitor the toxicity of pit lake³⁴ waters during *in situ* remediation trials (Neil *et al.*, 2009).

Microalgae, *Chlorella* spp. among them, although not specifically *C. protothecoides* or *A. protothecoides*, are also cultivated as feedstock for mariculture (Brown and Jeffery, 1992).

In similar fashion, although dietary supplements containing *Chlorella* spp. are widely available in the U.S. (Day *et al.*, 2009) and *Chlorella* continues to appear in the Utah Natural Products Alliance (UNPA) 'New Old Dietary Ingredients List' from the list's first release in 1997³⁵ to its current revised form, dated October 29, 2011 (UNPA, 2011), neither *C. protothecoides* nor *A. protothecoides* has been specified or documented as a species of use. *Chlorella* species *C. vulgaris* and *C. pyrenoidosa* have been and are currently used as foods and ingredients in dietary supplements. Electronic searches of the U.S. and European regulatory websites and the trade and scientific literature have revealed documentation for oil and high-lipid flour derived from *C. protothecoides* (or *A. protothecoides*) for use in human foods (FDA, 2012) (FDA, 2013).

³² Biomass requirements include the efficient production of specific lipid content and profiles

³³ National Pulp Mills Research Program, Commonwealth Scientific and Industrial Research Organisation (CSIRO) Division of Coal and Energy Technology in Australia

³⁴ Lake formed from surface mining or mining subsidence that has filled with surface and/or ground water.

³⁵ Purpose of the list was to identify those dietary ingredients that had been marketed in the U.S. prior to implementation of *Dietary Supplement Health and Education Act of 1994* (DSHEA, 1994) February 18, 2014

2.2. Proposed use or uses

WAP, a pale yellow to green high protein powder composed of the dried biomass of the green microalgae *Chlorella protothecoides* S106, will be used as a protein source, analogous to soy- and animal-based proteins, in a variety of foods.² The levels of intended use range from 10,000 ppm³⁶ (µg/g) in items such as processed vegetable juices, salad dressings, milk chocolate candies, and frozen dairy products to 160,000 ppm in protein powder, textured vegetable protein, and soy-based liquid meal replacements or supplements.

2.3. Mechanism of action

WAP will be used as protein source, analogous to soy- and animal-based proteins, in a variety of conventional foods. As a replacement source of macronutrients and energy, the proteins, lipids, and carbohydrates found in WAP will be digested through the same normal physiological processes by which other plant materials common to the human diet are digested and utilized.

2.4. Regulatory status

WAP from the dried biomass of *C. protothecoides* S106 has not been approved for use in food by FDA,³⁷ FEMA,³⁸ or USDA.³⁹ Algal oil derived from *C. protothecoides* S106, however, holds GRAS status (GRAS Notification 000384⁴⁰) for use as a food oil in a variety of foods excluding meat and poultry products. High-lipid algalin flour (HLAF) from *C. protothecoides* S106 (cultured under nitrogen depleted conditions) also holds GRAS status (GRAS Notification 000469⁴¹) for use as a partial replacement for cream, milk, eggs/egg yolks, and/or butter/shortening in baked goods, beverages, dairy and egg products, sauces, gravies, margarines, salad dressings, and soups.

In Europe, according to the Novel Food Regulation (EC) N° 258-97, neither WAP nor HLAF is a novel food; both are traditional food ingredients (TNO, 2012). The EU's Scientific Committee on Food does not have an established Acceptable Daily Intake (ADI) for HLAF in humans. In Australia and New Zealand, Whole Algalin Flour (both high-lipid and high-protein) is not considered a novel or non-traditional food based on the long history of food use for *Chlorella*. This information is published online, as a record of the Advisory Committee on Novel Foods (ANCF).⁴²

³⁶ ppm = parts *per* million

³⁷ FDA = United States Food and Drug Administration

³⁸ FEMA = Flavor and Extract Manufacturer's Association

³⁹ USDA = United States Department of Agriculture

⁴⁰ <<http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=384>>; site accessed October 11, 2013.

⁴¹ <<http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=469>>; site accessed October 11, 2013.

⁴² <http://www.foodstandards.gov.au/industry/novel/novelrecs/Documents/Novel%20Foods_mar2013%20Update.pdf>; site accessed December 17, 2013.

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3. DESCRIPTION, SPECIFICATIONS AND MANUFACTURING PROCESS

3.1. Description and Specifications

WAP is a pale yellow to green high protein powder composed of the dried biomass of the microalgae *Chlorella protothecoides* S106. The physical and chemical properties of WAP are provided in Table 3. The specifications for WAP are provided in Table 4. The amino acid profiles for three lots of WAP are provided in Table 5.

Table 3. Physical and chemical properties of WAP

Characteristic	Value
Synonyms	Algal protein, Algalin powder, Algalin protein, Whole algal protein, Whole algalin protein, High protein algal flour
Appearance	Pale yellow to green powder/flake
Moisture Content	≤ 10 %
Fiber Content	5 – 25 %
Ash Content	< 10 %
Protein Content	40 – 75 %
Fat Content	5 – 25 %

WAP= Whole Algal Protein

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Table 4. Specifications for WAP

Analysis	Method	Specification	Batch Analysis Results (<i>n</i> = 3)	
			Range	Mean
Appearance	C-M-00023-000 Appearance ^a	Pale yellow to green powder/flake	Conforms	Conforms
Moisture Content	AOAC 930.15	≤ 10 %	4.8 – 8.0 %	6.1 %
Fiber Content	AOAC 991.43	5 – 25 %	10.3 – 20.3 %	16.3 %
Ash Content	AOAC 942.05	< 10 %	7.5 – 8.7 %	7.9 %
Protein Content	AOAC 990.03	40 – 75 %	50.0 – 56.4 %	52.4 %
Fat Content	AOAC 954.02	5 – 25 %	15.0 – 16.6 %	15.8 %
Sucrose	AOAC 980.13	0 – 10 %	3.9 – 6.3 %	5.4 %
Heavy metals				
Lead	EPA 3050/6020 USP 730	< 0.5 ppm	<0.01 – 0.039 ppm	0.026 ppm
Arsenic	EPA 3050/6020 USP 730	< 0.2 ppm	0.031 – 0.24 ppm	0.101 ppm
Mercury	EPA 3050/6020 USP 730	< 0.1 ppm	< 0.005 ppm	< 0.005 ppm
Cadmium	EPA 3050/6020 USP 730	< 0.1 ppm	< 0.001 – 0.0047 ppm	0.003 ppm
Chromium	EPA 3050/6020 USP 730	< 2 ppm	0.044 – 0.367 ppm	0.174 ppm
Cobalt	EPA 3050/6020 USP730	< 0.1 ppm	<0.01 – 0.046 ppm	0.032 ppm
Microbiological Limits				
Aerobic Plate Count	AOAC 990.12	< 5000 cfu/g	< 10 – 2700 cfu/g	907 cfu/g
Coliform	AOAC 966.24	≤ 5 cfu/g	< 3 cfu/g	< 3 cfu/g
<i>E. coli</i>	USP 32, NF 27, 2009	Negative in 10 g	Negative	Negative
Staphylococci	USP 32, NF 27, 2009	Negative in 10 g	Negative	Negative
Salmonella	AOAC 2004.03	Negative in 25 g	Negative	Negative
<i>Pseudomonas aeruginosa</i>	USP 32, NF 27, 2009	Negative in 10 g	Negative	Negative
Yeast	Chapter 18, FDA- BAM, 7 th ed.	< 100 cfu/g	< 10 cfu/g	< 10 cfu/g
Mold	Chapter 18, FDA- BAM, 7 th ed.	< 100 cfu/g	< 10 – 10 cfu/g	< 10 cfu/g

^a C-M-007-00 Appearance (Solazyme internal method)

AOAC = Association of Official Analytical Chemists; cfu = colony-forming units; EPA = Environmental Protection Agency; FDA-BAM = Food and Drug Administration Bacteriological Analytical Manual; g = gram; *n* = number; WAP = Whole Algal Protein; ppm = parts *per* million; USP = United States Pharmacopeia

Table 5. Amino acid profile (g/100 g total protein) for WAP ($n = 3$ lots) compared with two *Chlorella* species and egg and soybean

Amino Acid	HPF358	HPF362	HPF378	Mean ($n = 3$)	<i>C. vulgaris</i> ^a	<i>C. pyrenoidosa</i> ^b	Egg ^a	Soybean ^a	FAO ^c
Methionine ^d	2.2	1.6	1.6	1.8	2.2	2.1	3.2	1.3	2.3 ^f
Cysteine	1.8	1.4	0.9	1.4	1.4	1.6	2.3	1.9	See ^f
Lysine ^d	6.6	4.9	5.5	5.7	8.4	4.9	5.3	6.4	4.8
Phenylalanine ^d	3.2	2.8	3.7	3.2	5.0	5.5	5.8	5.0	4.1 ^g
Leucine ^d	7.8	5.5	7.6	7.0	8.8	5.6	8.8	7.7	6.1
Isoleucine ^d	2.8	1.6	2.7	2.3	3.8	3.7	6.6	5.3	3.0
Threonine ^d	5.0	3.5	4.3	4.3	4.8	4.9	5.0	4.0	2.5
Valine ^d	5.0	2.9	4.3	4.1	5.5	5.2	7.2	5.3	4.0
Histidine ^e	3.2	1.6	2.0	2.2	2.0	3.0	2.4	2.6	1.6
Arginine ^e	7.0	22.0	26.8	18.6	6.4	13.4	6.2	7.4	
Glycine	6.6	4.5	5.1	5.4	5.8	5.5	4.2	4.5	
Aspartic Acid	8.0	8.1	9.6	8.5	9.0	7.1	11.0	1.3	
Serine	5.4	4.1	4.3	4.6	4.1	5.1	6.9	5.8	
Glutamic Acid	45.6	20.6	18.3	28.2	11.6	10.3	12.6	19.0	
Proline	17.6	6.5	7.1	10.4	4.8	5.6	4.2	5.3	
Hydroxyproline	0.6	0.2	0.4	0.4	NA	0.52	NA	NA	
Alanine	11.4	9.4	8.5	9.8	7.9	6.2	---	5.0	
Tyrosine	2.4	2.0	2.8	2.4	3.4	4.7	4.2	3.7	See ^g
Tryptophan ^d	0.8	0.8	1.1	0.9	2.1	0.49	1.7	1.4	0.7

^a (Brown and Jeffery, 1992; Becker, 1994; Becker, 2007; Christaki *et al.*, 2011) (Please note: CSIRO culture CS-41 is *C. pyrenoidosa* Chick. The strain was mis-identified as *C. protothecoides* at isolation in 1947; the error was recognized and corrected in 2010. (<<http://www.marine.csiro.au/algaedb/FMPro?-db=cmarc%20database.fp5&-format=record%5fdetail.htm&-lay=everything&-sortfield=genera%20plus%20species&-op=eq&Culturestatus=Published&-op=eq&CS%20Number=cs-41&-max=15&-recid=34970&-find>>; site accessed October 24, 2013)); ^c FAO, 2013 (Based on the amino acid requirements for children. Except for isoleucine, maintenance requirements for adults are somewhat lower.); ^d Essential amino acids for humans; ^e Semi-essential amino acids (exogenous source required during periods of growth); ^f Methionine + cysteine (the sulfur amino acids); ^g Phenylalanine + tyrosine (the aromatic amino acids) CSIRO = Commonwealth Scientific and Industrial Research Organisation of Australia; FAO = Food and Agriculture Organization of the United Nations; n = number; NA = Not analyzed; WAP = Whole Algal Protein; n = number.

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Protein content in the two most commonly consumed *Chlorella* species, *C. vulgaris* and *C. pyrenoidosa*, has been reported, respectively, at levels of 57% and 51-58% total dry matter (Becker, 2007). Beyond total protein content, the nutritional quality of protein⁴³ is based on the identity, relative presence, and availability of its amino acids (Becker, 2007). Compared with proteins from animal sources, proteins derived from microalgae often contain marginal levels of the (human) essential amino acid lysine and sulfur-containing amino acids methionine and cysteine (Kay, 1991). On the other hand, microalgal-derived proteins usually have better amino acid profiles when compared with proteins from vegetable sources (e.g., cereal grains and legumes) (Lee *et al.*, 1967; Kay, 1991). Overall, most microalgae contain the essential and semi-essential amino acids⁴⁴ and in general, compare favorably with the amino acid profiles of conventional food proteins (Becker, 2007). The amino acid profile of *C. vulgaris* (Table 5) compares favorably with egg, is somewhat better than soybean, and meets the FAO guideline profile for children (Becker, 2007; FAO, 2013). Although similar to *C. vulgaris*, the amino acid profile of *C. pyrenoidosa* (Table 5) is richer in arginine, deficient in leucine and tryptophan, and contains a marginal level of lysine (Brown and Jeffery, 1992; FAO, 2013). Compared with *C. vulgaris* and *C. pyrenoidosa*, WAP from *C. protothecoides* contains marginal levels of the essential amino acids valine and tryptophan, is deficient in isoleucine, but is a rich source of glutamic acid, proline, and alanine (Table 5).

Pheophorbides (Pheides) are naturally occurring degradation products of chlorophyll that form during 'de-greening' events (Jassby, 1988). Ingestion of pheophorbide-A (PheideA) is known to induce photosensitive dermatitis in humans (Jitsukawa *et al.*, 1984; Jassby, 1988). When Solazyme's ground pale yellow-to-green high-protein *C. protothecoides* algal biomass⁴⁵ was assayed, the concentration for PheideA was determined to be 0.0334 mg/g (FDA, 2012; Szabo *et al.*, 2013), a concentration considerably lower than the limit of < 1.2 mg/g established by the Japanese Public Health Ministry in 1981 (Becker, 1994; FDA, 2012).

Algal and cyanobacterial toxins have a broad range of potencies. Ingestion of one or more of these toxins by a human can cause a range of effects from mild illness to death. Solazyme's high-protein *C. protothecoides* biomass was analyzed by high performance liquid chromatography with fluorescence or mass spectrometric detection for all groups of algal and cyanobacterial toxins that have been identified in the published literature and mentioned in international food regulations (Day *et al.*, 2009; FDA, 2012). Assayed toxins included amnesic shellfish poisoning toxins (domoic acid), paralytic shellfish poisoning toxins (*N*-sulfocarbamoyl toxins C1-4, B1, B2; decarbamoylgonyautoxins 1-4; gonyautoxins 1-4; decarbamoylsaxitoxin; saxitoxin; and neosaxitoxin), diarrhetic shellfish poisoning toxins (okadaic acid,

⁴³ Protein bioavailability from the whole cell WAP product was demonstrated to be 87.4 % in an unpublished study using a dynamic gastrointestinal (GI) model simulating the upper GI tract (TNO, 2012).

⁴⁴ The essential amino acids cannot be synthesized by humans or animals. The semi-essential amino acids histidine and tryptophan require an exogenous source during periods of growth.

⁴⁵ The *C. protothecoides* used in this material was produced through a process similar to that used in the production of WAP. The material studied by Day *et al.* (2009) and the subject of GRN000384 (FDA, 2012) has been confirmed by Solazyme to be the same material as that of the current GRAS.

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dinophysistoxins, pectenotoxins, yessotoxins, azaspiracides, and gymnodimines), neurotoxic shellfish poisoning toxins (brevetoxins), and the cyanobacterial toxins (microcystins MC-RR, -LR, -YR, -LA, -LF, and -LW), nodularin, anatoxin-a, cylindrospermopsins, and β -methylamino-L-alanine (BMAA). No toxins were detected in the high-protein biomass (Szabo *et al.*, 2013).⁴⁶

3.2. Manufacturing Process

WAP is manufactured by fermenting and harvesting cultures of *C. protothecoides* S106. A pure, clonally isolated culture is initially used to prepare a master seed bank from which working seed vials are prepared. Three samples from the master and each working seed bank are characterized by molecular genotyping to demonstrate that they are genetically identical (*i.e.*, 100% homology between the six chromosomal footprints and 100% homology between their 23S ribosomal deoxyribonucleic acid (DNA) sequences).⁴⁷ For a production lot, a cryo-preserved working seed vial is thawed and the contents used to inoculate a flask culture, which is transferred into larger flasks at mid-log phase, and then to standard, industrial seed fermenters. Throughout the aseptic fermentation process, pH, temperature, agitation and aeration rates are controlled, and glucose (carbon source) and nutrients are added (APPENDIX 1).

Protein production is favored during fermentation by ensuring that glucose is available as the carbon source and inorganic nitrogen is not restricted. Following fermentation, the cells are washed to remove soluble medium components and concentrated by centrifugation. The separated cells are then inactivated by pasteurization (85 °C, 1 minute). After concentration, the pH is adjusted close to neutral (6.0-8.0) and food-grade antioxidants (FDA-approved) are added. The biomass, primarily whole cells, is then dried, and optionally milled (to reduce particle size if needed), and packaged. If needed, food grade flow agents (APPENDIX 1) may be added to assist processing through drying and packaging; at present the use of such agents is not part of the standard process. All ingredients used during manufacture are safe and suitable (APPENDIX 1).⁴⁸ A graphical depiction of the manufacturing process is presented in Figure 2.

The final product, WAP, is available in quantities of 15 – 25 kg packed in a product bag and stored under cool dry conditions (< 25 °C), away from direct sunlight.

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⁴⁶ Limits of detection ranged from 0.1–2.5 $\mu\text{g/g}$.

⁴⁷ When tested, the six 23S ribosomal DNA sequences also demonstrated 100% identity to the 23S reference sequence for the original *C. protothecoides* S106 isolate.

⁴⁸ Regarding labeling concerns related to the eight major food allergens, two of the antioxidants that may be added to the finished product were derived from soy.

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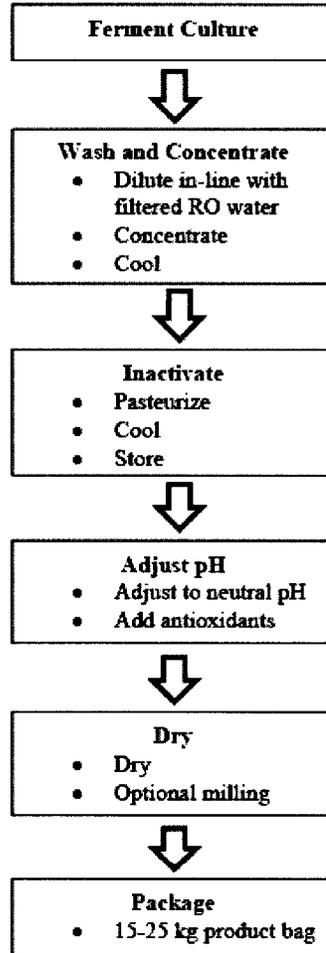


Figure 2. WAP production schematic

3.3. Stability

WAP is stable for 18 months when stored at -20 °C (Table 6) or at controlled room temperature, 23 ± 3 °C (Table 7). WAP is stable for three months when stored at 40 °C (Table 8); after three months at this temperature, all samples bleached. WAP was stored in sealed foil pouches for all stability evaluations.

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Table 6. Stability of WAP stored at -20 °C

Lot	Appearance (Pale green powder)	Protein Content ^a (≥ 40%)	Moisture (≤ 10%)
BMP 358			
0 month	Conforms	50.4	6.8
6 months	Conforms	50.0	6.6
12 months	Conforms	49.9	7.0
18 months	Conforms	48.0	5.9
BMP 362			
0 month	Conforms	50.8	6.4
6 months	Conforms	50.7	6.4
12 months	Conforms	50.3	6.5
18 months	Conforms	49.3	6.0

^a Dry basis adjusted; WAP = Whole Algal Protein

Table 7. Stability of WAP stored at 23 ± 3 °C

Lot	Appearance (Pale green powder)	Protein Content ^a (≥ 40%)	Moisture (≤ 10%)
BMP 358			
0 month	Conforms	50.4	6.8
3 month	Conforms	50.0	6.8
6 months	Conforms	50.0	7.3
9 months	Conforms	50.3	8.2
12 months	Conforms	50.5	8.2
18 months	Conforms	48.0	7.2
BMP 362			
0 month	Conforms	50.8	6.4
3 month	Conforms	50.7	6.6
6 months	Conforms	50.6	6.8
9 months	Conforms	51.5	8.3
12 months	Conforms	51.6	7.4
18 months	Conforms	50.6	8.2

^a Dry basis adjusted; WAP = Whole Algal Protein

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Table 8. Stability of WAP at 40 °C

Lot	Appearance (Pale green powder)	Protein Content ^a (≥ 40%)	Moisture (≤ 10%)
BMP 358			
0 month	Conforms	50.4	6.8
1 month	Conforms	50.2	6.4
3 month	Conforms	50.2	5.9
6 months	<i>Bleached</i> ^b	49.8	5.3
9 months	<i>Bleached</i>	50.6	5.8
12 months	<i>Bleached</i>	49.9	5.4
BMP 362			
0 month	Conforms	50.8	6.4
1 month	Conforms	50.5	6.9
3 month	Conforms	50.8	6.2
6 months	<i>Bleached</i>	50.6	5.6
9 months	<i>Bleached</i>	51.3	6.6
12 months	<i>Bleached</i>	50.5	5.6

^a Dry basis adjusted; ^b Use of bolded *italics* indicates parameters outside of specifications (*i.e.*, “Bleached”, rather than “pale green” appearance); WAP = Whole Algal Protein

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4. ESTIMATED DAILY INTAKE

The intake profile (amount and frequency) by individuals in USDA's What We Eat in America (WWEIA) Continuing Survey of Food Intakes by Individuals 2003-2004 (Dwyer et al., 2003)⁴⁹ was used to calculate the estimated daily intake (EDI) of WAP for individuals consuming the food groups selected for the addition of WAP *per* this GRAS evaluation (*i.e.*, "eaters only"). The individual foods selected for addition of WAP are provided in Appendix 2. WAP will be added only to foods for which a standard of identity does not exist.

The means and 90th percentile EDIs were calculated only for WAP intake following addition of WAP to the selected food groups. The means and 90th percentile EDIs were not calculated for current WAP intake from natural sources as no information regarding current intakes of WAP from natural sources was discovered during a comprehensive search of the published literature. WAP added to the selected foods at the levels specified in Appendix 2 would provide a mean and 90th percentile WAP consumption of 2.32 and 5.56 g/day, respectively (Table 9.).

All food categories designated by Solazyme have been utilized in the calculations as appropriate; however, certain categories designated by Solazyme may contain foods for which a standard of identity exists. We note that an ingredient that is lawfully added to food products may be used in a standardized food only if it is permitted by the applicable standard of identity. Solazyme confirms that WAP will be added only to foods for which a standard of identity does not exist.

Table 9. Predicted intake of WAP following supplementation of selected foods at the indicated levels (Appendix 2) for individuals consuming selected supplemented foods

WAP intake from:	Per User (mg/day)	
	Mean	90 th Percentile
Possible maximum consumption with WAP as an added ingredient to food	2319	5562

WAP = Whole Algal Protein

5. ABSORPTION, DISTRIBUTION, METABOLISM AND ELIMINATION (ADME)

The proteins, lipids, and carbohydrates found in WAP (a high protein *Chlorella protothecoides* S106 powder) are expected to be digested, absorbed, metabolized and excreted through the same normal physiological processes by which other plant materials common to the human diet are digested.

⁴⁹ USDA (2006) What We Eat In America, NHANES 2003-2004; Documentation and Data Files. U.S. Department of Agriculture, Agriculture Research Service; <http://www.ars.usda.gov/Services/docs.htm?docid=15044>; site visited October 17, 2013.

6. SAFETY EVALUATION

6.1. Acute Studies

No acute toxicity studies related to *C. protothecoides* or *A. protothecoides* were discovered in the scientific literature.

6.2. Short Term Repeated-Dose Studies

In a 28-day repeated-dose toxicity study (Day *et al.*, 2009) HSD:SD[®] rats ($n = 10/\text{sex}/\text{group}$) were each provided diets *ad libitum* containing 0 ppm (placebo control), 25,000 ppm (low-dose), 50,000 ppm (mid-dose) and 100,000 ppm (high-dose) ground, yellow, high-lipid⁵⁰ *C. protothecoides* S106 biomass. This high-lipid material was derived from the same strain of *C. protothecoides* as WAP, but under nitrogen-limited conditions. The test diets for the Day *et al.* (2009) study were formulated using the AIN-93G Rodent Diet (Research Diets, Inc., New Brunswick, NJ) as the basal diet to which sufficient test substance was added to achieve the target concentrations and to ensure comparable fat, protein and carbohydrate content across dose and control groups. The low-, mid-, and high-dose diets were equivalent to 1794, 3667, and 7557 mg/kg bw⁵¹/day, respectively, in males and 1867, 3918, and 8068 mg/kg bw/day, respectively, in females. Following the treatment period, the rats from each group were euthanized on Day 31 (males) or Day 32 (females). This study was performed in compliance with OECD⁵² Guidelines⁵³ and under conditions of Good Laboratory Practice (GLP).⁵⁴

The daily administration of the ground, yellow, high-lipid *C. protothecoides* biomass material at dietary concentrations up to 100,000 ppm in the feed was well-tolerated by the rats. Consumption did not affect health or growth as measured by viability, appearance, behavior, body weight, body weight gain, food consumption, or food efficiency. No treatment-related effects were identified in the ophthalmology, urinalysis, hematology, clinical chemistry, gross pathology, organ weights, or histopathology of animals in any group. Although statistical significance was shown for several parameters, none were attributable to ingestion of the test substance because the changes were noted only sporadically, did not demonstrate a dose-response relationship, were within the ranges historically observed in the age and strain of rats used in this study, and/or were also observed in the control group. These observations are summarized below:

(a) One control female died from suspected accidental overdose of anesthesia on Day 29 during orbital sinus bleeding. This mortality was an isolated incident and not related to the test substance. (b) Incidental findings during clinical observation included a scab on the head of one mid-dose male (Days 2-6) and an abrasion on the nose of one low-dose female (Day 3). (c) A

⁵⁰ 48% lipid; 6% protein.

⁵¹ bw = body weight

⁵² OECD = Organisation for Economic Co-operation and Development

⁵³ OECD Guidelines for the Testing of Chemicals, Section 4 (Part 407): Health Effects, *Repeated Dose 28-day Oral Toxicity Study in Rodents* (1995).

⁵⁴ OECD Principles of Good Laboratory Practice (as revised in 1997), OECD Environmental Health and Safety Publication, Series on Principles of Good Laboratory Practice and Compliance Monitoring – Number 1. Environment Directorate, Paris 1998 and US FDA GLP: 21 CFR §58, 1987.

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minor unilateral observation was also made in one control female during ophthalmologic examination on Day 25. The finding was confined to a single animal and was not associated with the test substance. (d) A small albeit statistically significant increase in food consumption was observed in high-dose males during Week 2 ($P < 0.05$) compared to the control group. Although food consumption by high-dose males did not differ significantly from the control group during the other three weeks, overall food consumption (Days 0-28) was also statistically higher for high-dose males compared to the controls ($P < 0.05$). Because increased consumption was slight and not accompanied by corresponding changes in body weight or food efficiency, the finding was not toxicologically significant and not necessarily related to the test substance. (e) A statistically significant decrease in mean food efficiency was reported in mid-dose males during Week 3 ($P < 0.05$) compared to control males. This decrease was short term and, therefore, was determined to be incidental and not related to the test substance. (f) Statistically significant decreases in mean corpuscular hemoglobin concentration (MCHC) and absolute basophil concentrations were noted for the males of the high-dose and low-dose groups, respectively, compared to the control group ($P < 0.05$ for both). Because these Day 29 hematology findings were not accompanied by any other clinical or histopathologic change and no dose-dependent relationship was demonstrated, the findings were not attributable to treatment. (g) A significant increase in the absolute large unstained cell concentration in mid-dose group females compared to the control group ($P < 0.05$) was not toxicologically relevant because the change was sporadic (not present at Day 29) and did not demonstrate a dose-dependent relationship. (h) Clinical chemistry results revealed statistically significant increases in creatinine in mid-dose males and triglycerides in high-dose males on Day 15 and blood urea nitrogen in mid-dose males on Day 29 compared to the control group ($P < 0.05$ for all). Cholesterol in high-dose females was significantly elevated compared to the control group ($P < 0.05$) on Day 15. Because these findings did not present with consistency, dose-response relationship, or corresponding clinical or histopathological changes, they were not related to the test substance. (i) Clinical chemistry results from Days 15 and 29 revealed statistically significant increases in alkaline phosphatase in high-dose group males ($P < 0.05$ for both). These findings did not demonstrate a dose-response relationship and were not present in females, nor were they accompanied by corresponding clinical or histopathologic change, nor were there corresponding changes in liver or kidney weight; they were, therefore, not found to be toxicologically relevant. (j) Findings for Day 29 urinalysis included a statistically significant increase in specific gravity and a decrease in urine volume in mid-dose females compared to controls ($P < 0.05$ for both). These changes were not dose-related nor were they accompanied by clinical or histopathologic change, nor was liver or kidney weight affected. The changes were not treatment related. (k) Macroscopic observation revealed a statistically significant increase in absolute adrenals ($P < 0.05$), adrenals-to-body ($P < 0.01$) and adrenals-to-brain ($P < 0.05$) weights in the mid-dose males compared to the control group values. Due to the absence of clinical or histopathological changes, these observations were incidental.

Under the conditions of this study, the no-observed-adverse-effect level (NOAEL) for the ground, yellow, high-lipid *C. protothecoides* biomass in the diet, was 100,000 ppm, the highest dietary concentration provided in the study, which corresponds to a dietary NOAEL of 7557 mg/kg bw/day in male rats and 8068 mg/kg bw/day in female rats.

The findings of an unpublished 28-day trial of dietary *C. protothecoides* (strain not specified) in the Syrian golden hamster (sex not specified; $n = 15/\text{group}$) (Harding and Jones, 2008) corroborated the findings of the published Day *et al.* (2009) study. Control animals received a hyperglycemic-hypercholesterolemic diet *ad libitum*; the treatment groups received the same diet supplemented with 2.5% or 5.0% (w/w)⁵⁵ *C. protothecoides* (equivalent to ~3000 or ~6000 mg/kg bw/day, respectively). No statistically significant effect was reported in body weight, food intake, body composition (e.g., percentage body fat), or plasma triglycerides, cholesterol, protein, or albumin for the treatment groups compared to the control group. Compared to the control group, plasma glucose was significantly reduced in both low- and high-dose groups ($P < 0.05$ for both), but without affecting triglycerides or total cholesterol. Although plasma insulin concentrations for the treatment group did not differ significantly compared to the control group, plasma insulin in the 2.5% group was significantly elevated compared to plasma insulin in the 5.0% group ($P < 0.05$). Oxygen consumption was also significantly greater in the 2.5% group than in the control group ($P < 0.05$), but without affecting production of carbon dioxide. The authors concluded that *C. protothecoides* in a hyperglycemic-hypercholesterolemic diet decreased plasma glucose independent of plasma insulin and further determined that “consumption of *C. protothecoides* at 2.5% and 5% of total diet (w/w) [~3000 and ~6000 mg/kg bw/day, respectively] appeared to be safe as there was no difference between groups in the liver production total plasma protein or albumin” (Harding and Jones, 2008).

In summary, although statistically significant effects were noted for several endpoints in a 28-day repeated-dose study in rats (Day *et al.*, 2009), these were not related to administration of the test substance (a ground, yellow, high-lipid *C. protothecoides* S106 biomass material) or were not toxicologically relevant. Under the conditions of the study, the NOAEL for the high-lipid *C. protothecoides* biomass in the diet was 100,000 ppm, the highest dietary concentration provided in the study, which corresponds to a dietary NOAEL of 7557 mg/kg bw/day in male rats and 8068 mg/kg bw/day in female rats. In a corroborative unpublished 28-day repeated-dose study in hamsters, consumption of a *C. protothecoides* up to ~6000 mg/kg bw/day was determined by the authors to be safe (Harding and Jones, 2008). The short-term repeated-dose toxicity studies in rodents are summarized in Table 10.

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⁵⁵ w/w = weight/weight

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Table 10. 28-Day short term repeated-dose toxicity studies in rodents

Duration	Species (#/dose group)	Dose/Route	Results/Notes	Reference
28 days	Rat (10M/10F)	0, 25,000, 50,000 and 100,000 ppm high-lipid <i>C. protothecoides</i> S106 biomass in diet	Dietary ground, yellow, high-lipid <i>C. protothecoides</i> biomass was well-tolerated; NOAEL was 7557 mg/kg bw/day in males and 8068 mg/kg bw/day in females. No reported adverse effects.	Day <i>et al.</i> (2009)
28 days	Syrian golden hamster (15/group; sex not specified)	0, 2.5% and 5.0% “relatively high-lipid biomass” <i>C. protothecoides</i> in diet (equivalent to 0, ~3000, and ~6000 mg/kg bw/day)	Plasma glucose significantly reduced in low- and high-dose groups compared to the control ($P < 0.05$ for both). Plasma insulin for treatment groups did not differ significantly compared to control, but plasma insulin in low-dose group was significantly elevated compared to high-dose group ($P < 0.05$). Oxygen consumption significantly greater in low-dose group than in control group ($P < 0.05$). Consumption of <i>C. protothecoides</i> at up to ~6000 mg/kg bw/day was determined by the authors to be safe.	Harding and Jones (2008), unpublished report; Corroborative

C. Protothecoides = *Chlorella protothecoides*; F = Female; M = Male; NOAEL = No-observed-adverse-effect level; ppm = parts per million; # = number; bw = body weight

6.3. Subchronic Studies

6.3.1. WAP subchronic repeated-dose toxicity study

In a 13-week repeated-dose subchronic toxicity study (Szabo *et al.*, 2013), HSD:SD[®] rats ($n = 10$ /sex/group) were provided *ad libitum* diets containing 0 ppm (Group 1 placebo control), 25,000 ppm (Group 2), 50,000 ppm (Group 3) and 100,000 ppm (Group 4) WAP (equivalent to 0, 1177, 2416, and 4805 mg/kg bw/day, respectively in males and 0, 1444, 2700, and 5518 mg/kg bw/day, respectively, in females), a pale yellow to green high protein powder composed of the dried, milled biomass of *C. protothecoides* S106.⁵⁶ The test diets were formulated using the DIO Rodent[®] basal diet to which sufficient WAP was added to achieve the target concentrations and to ensure comparable fat, protein and carbohydrate content across dose and control groups. Following the treatment period, the rats from each group were terminated on Day 92 (males) or Day 93 (females). The study was performed under GLP⁵⁴ and in compliance with OECD Guidelines.⁵⁷ The study protocol was reviewed by the Institutional Animal Care and Use Committee (IACUC) of PSL⁵⁸ (Dayton, NJ), which is fully accredited by Accreditation of Laboratory Animal Care International (AAALAC International) (Accredited Unit No. 000939). The subchronic repeated-dose toxicity study for WAP in rodents is summarized in Table 11.

⁵⁶ The test material used in this study (assayed to contain 59.9% protein) differs from the product material in that the test material was milled (> 30% cells disrupted); the product material is whole cell.

⁵⁷ OECD Guidelines for Testing of Chemicals and Food Ingredients, Section 4 (Part 408): Health Effects, *Repeated Dose 90-Day Oral Toxicity Study in Rodents* (1998).

⁵⁸ PSL = Product Safety Labs

Daily exposure to WAP at dietary concentrations up to 100,000 ppm was well-tolerated by the rats in the 13-week study. No test substance-related mortalities occurred during the study period. Consumption of WAP was not found to affect health or growth as measured by viability, condition, behavior, body weight, body weight gain, food consumption, or food efficiency. No treatment-related effects were identified in the ophthalmology, urinalysis, hematology, clinical chemistry, gross pathology, absolute or relative organ weights, or histopathology of animals in any group. Although statistical significance was shown for several parameters, none were attributable to ingestion of WAP because the changes were incidental or noted only sporadically, were not accompanied by corresponding clinical or histopathologic changes, did not demonstrate a dose-dependent relationship, and/or were also observed in the control group. These observations are summarized below.

(a) One Group 1 (control) male died on Day 27 after receiving anesthesia for a teeth clipping procedure to address maloccluded upper incisors. No findings, including macroscopic or microscopic pathology, were discovered that explained the early mortality; this event was incidental and not related to the test substance. (b) Mean daily body weight gains for Group 4 males on Days 77-84 ($P < 0.01$), Group 3 females on Days 14-21 ($P < 0.01$), and Group 4 females on Days 49-56 ($P < 0.05$) were significantly increased compared to their corresponding control groups. These findings were incidental and not attributable to test substance exposure. (c) A statistically significant decrease in mean daily food consumption compared to the control group was observed in Group 2 males on Days 7-14 ($P < 0.05$), 21-35 ($P < 0.01$), 35-42 ($P < 0.05$), 42-49 ($P < 0.01$), 56-63 ($P < 0.05$), 63-77 ($P < 0.001$) and overall, Days 0-91 ($P < 0.01$); in Group 3 males on Days 63-77 ($P < 0.05$); and in Group 4 males on Days 7-14 ($P < 0.05$), 21-35 ($P < 0.05$), 49-56 ($P < 0.05$), 56-63 ($P < 0.01$), 63-70 ($P < 0.05$), 70-77 ($P < 0.01$), and overall, Days 0-91 ($P < 0.05$). In comparison, mean food efficiency significantly increased only in Group 4 males on Days 77-84 ($P < 0.01$); food efficiency in all other male treatment groups was comparable to the control group. Because decreased consumption was not dose-dependent, was minimal on an individual basis, and was not accompanied by decreases in body weight or food efficiency, the findings were not adverse effects. (d) Among females, mean daily food consumption significantly decreased only in Groups 3 and 4 and only on Days 28-35 ($P < 0.05$ and $P < 0.01$, respectively) while mean food efficiency increased significantly in Group 3 on Days 14-21 ($P < 0.01$) and Group 4 on Days 49-56 ($P < 0.05$), compared to the control group. Statistically significant changes in mean food consumption and efficiency observed among the female groups were sporadic and not dose-related and were therefore determined to be unrelated to treatment. (e) Statistically significant changes in urinalysis parameters on Day 91 were limited to decreased urine volume and pH in Group 2 males ($P < 0.05$ for both), compared to controls. These changes were not toxicologically relevant because they were not associated with any corresponding clinical or histopathologic change. (f) Hematology results for Day 91 revealed a statistically significant decrease in the absolute lymphocyte concentrations in Group 2 males compared to the control group ($P < 0.05$). Because this finding did not demonstrate a dose-dependent relationship and was not accompanied by corresponding clinical or histopathologic changes, it was not toxicologically relevant. (g) Clinical chemistry results from Day 91 revealed a statistically significant decrease in total cholesterol in Group 4 males, compared to control

males ($P < 0.05$). This finding did not demonstrate a dose-dependent relationship,⁵⁹ nor was it accompanied by corresponding clinical or histopathologic changes; it was determined, therefore, not to be toxicologically relevant. (h) A soft, tan vascularized mass (12 x 6 x 9 mm) in the right epididymis in a Group 4 male on Day 92 corresponded histologically to a unilateral moderately sized sperm granuloma; the mass was of spontaneous origin. (i) A firm, tan, cervical subcutaneous, lobulated mass (10 x 10 x 5 mm) in a Group 1 female histologically identified as a mammary gland adenocarcinoma; an extrahepatic nodule (10 x 7 x 8 mm) on the dorsal aspect of the liver in another Group 1 female found to be composed of liver of normal morphology; and a lobulated diaphragmatic hernia in the liver in a Group 3 female histologically identified as a hepatodiaphragmatic nodule were each were spontaneous and unrelated to the test substance.

In summary, although statistically significant effects were reported for several endpoints, none were attributable to ingestion of WAP because the changes were incidental or noted only sporadically, were not accompanied by corresponding clinical or histopathologic changes, did not demonstrate a dose-dependent relationship, and/or were also observed in the control group. Under the conditions of this study, the NOAEL for WAP in the diet was 100,000 ppm, the highest dietary concentration provided in the study, which corresponded to a dietary NOAEL of 4805 mg/kg bw/day in male rats and 5518 mg/kg bw/day in female rats.

6.3.2. HLAf (high-lipid algalin flour) subchronic repeated-dose toxicity study

In a 13-week repeated-dose subchronic toxicity study (Szabo *et al.*, 2012; FDA, 2013), HSD:SD[®] rats ($n = 10/\text{sex}/\text{group}$) were provided *ad libitum* diets containing 0 ppm (Group 1 placebo control), 25,000 ppm (Group 2), 50,000 ppm (Group 3) and 100,000 ppm (Group 4) HLAf (equivalent to 0, 1249, 2478, and 4807 mg/kg bw/day, respectively in males and 0, 1413, 2739, and 5366 mg/kg bw/day, respectively, in females). The test diets were formulated using the DIO Rodent[®] basal diet to which sufficient HLAf (a golden yellow high-lipid powder composed of the milled dried biomass of *C. protothecoides* S106) was added to achieve the target concentrations and to ensure comparable fat, protein and carbohydrate content across dose groups. Following the treatment period, the rats from each group were terminated on Day 93 (males) or Day 94 (females). This study was performed under GLP and in compliance with OECD Guidelines as described previously in Section 6.3.1. The protocol was reviewed by PSL IACUC as described previously in Section 6.3.1. The subchronic repeated-dose toxicity study for HLAf in rodents is summarized in Table 11.

Daily consumption of HLAf at dietary concentrations up to 100,000 ppm was well-tolerated by the rats in the 13-week study and did not affect health or growth as measured by viability, condition, behavior, body weight, body weight gain, food consumption or food efficiency. Further, no treatment-related effects were identified in the ophthalmology, urinalysis, hematology, clinical chemistry, gross pathology, organ weights or histopathology of animals in any group. Although statistical significance was shown for several parameters, none were attributable to ingestion of HLAf because the changes were within the ranges historically

⁵⁹ Although total cholesterol values in Group 2 and Group 3 males were suggestive of a dose-response trend, the values for these groups did not vary significantly from Group 1 control values; in addition, the individual values for these three groups largely overlapped.

observed in the age and strain of rats used in this study, did not demonstrate a dose-dependent relationship, lacked any clinical or histopathologic correlation, were noted only sporadically, and/or were also observed in the control group. These observations are summarized below:

(a) A firm, round, subcutaneous lesion on the left side of the neck (cranial) area in one Group 2 female was identified during pathological evaluation as an adenocarcinoma of mammary gland origin. The lesion was an isolated finding limited to this one female. (b) A statistically significant decrease in food consumption was observed in Group 4 males on Days 14-21, 35-56, and 84-91 ($P < 0.05$ for all) and overall, Days 0-91 ($P < 0.05$) as compared to the control group. Because decreased consumption occurred during three discrete weeks out of 13 weeks, was minimal on an individual basis regarding the Day 0-91 assessment and was not accompanied by decreases in body weight or food efficiency, the finding was not an adverse finding. (c) A statistically significant increase in mean food efficiency in Group 3 females on Days 21-28 ($P < 0.05$) and overall 0-91 Days ($P < 0.01$), compared to control females was not dose-related and was therefore not treatment-related. (d) A statistically significant decrease in mean daily food consumption was reported for Group 4 females compared to the control group ($P < 0.01$) on Days 63-70. This decrease was short term and, therefore, was incidental and not related to the test substance. (e) A vitreal hemorrhage obscuring the optic nerve was reported in the right eye of a Group 1 (control) female on Day 90. The finding was interpreted to result from a sporadically occurring retinal vascular abnormality or hyaloid artery remnant. (f) Statistically significant increases in platelet concentration were noted for the females of Group 2 and Group 4 compared to the control group ($P < 0.05$ for all). Because these Day 91 hematology findings were not accompanied by any other clinical or histopathologic change and no dose-response relationship was demonstrated, the findings were not attributable to HLA-F administration. (g) A significant increase in the absolute reticulocytes in Group 3 females compared to the control group ($P < 0.05$) was not toxicologically relevant because the change was sporadic, was not accompanied by any other clinical or histopathologic change and not dose-dependent. (i) Clinical chemistry results from Day 91 revealed statistically significant increases in alkaline phosphatase in Group 2 and Group 3 males, decreased aspartate aminotransferase in Group 2 females, and an increase in total protein in Group 2 females compared to their respective control groups ($P < 0.05$ for all). These findings did not demonstrate a dose-response relationship, nor were they accompanied by corresponding clinical or histopathologic change; they were, therefore, not toxicologically relevant. (j) A firm, white lesion in the right kidney of a Group 4 male corresponded to a neoplasm having morphologic features consistent with a renal mesenchymal tumor. The tumor was an isolated finding, limited to this one male. (k) The enlarged, pale, mottled thymus noted in a Group 2 male lacked any histologic correlate and was spontaneous in origin. (l) In comparison to controls, Group 3 male relative adrenal-, brain-, and testis-to-body weight ratios were significantly decreased, while the kidney-to-brain weight ratio was significantly increased ($P < 0.05$ for all). In Group 2 and 3 females, absolute liver weights, liver-to-body weight and liver-to-brain weight ratios were significantly increased when compared to the control group ($P < 0.01$ for all Group 2 parameters; $P < 0.05$ for Group 3). All changes were incidental and not toxicologically relevant as no change demonstrated a dose-dependent relationship and none were found in the high-dose group.

In summary, although statistically significant effects were determined for several endpoints, none were attributable to ingestion of HLAf because the changes were within the ranges historically observed in the age and strain of rats used in this study, did not demonstrate a dose-dependent relationship, lacked any clinical or histopathologic correlation, were noted only sporadically, and/or were also observed in the control group. Under the conditions of this study, the NOAEL for HLAf in the diet is 100,000 ppm, the highest dietary concentration provided in the study, which corresponds to a dietary NOAEL of 4807 mg/kg bw/day in male rats and 5366 mg/kg bw/day in female rats.

Table 11. 90-Day subchronic repeated-dose toxicity studies in rodents

Duration	Species (#/dose group)	Dose/Route	Results/Notes	Reference
13 weeks	Rat (10M/10F)	0, 25,000, 50,000 and 100,000 ppm WAP in diet (equivalent to 0, 1177, 2416, and 4805 mg/kg bw/day, respectively in males and 0, 1444, 2700, and 5518 mg/kg bw/day, respectively, in females)	Dietary WAP was well-tolerated; NOAEL was 4805 mg/kg bw/day in males and 5518 mg/kg bw/day in females; No adverse effects were reported.	Szabo <i>et al.</i> (2013)
13 weeks	Rat (10M/10F)	0, 25,000, 50,000 and 100,000 ppm HLAf in diet (equivalent to 0, 1249, 2478, and 4807 mg/kg bw/day, respectively in males and 0, 1413, 2739, and 5366 mg/kg bw/day, respectively, in females)	Dietary HLAf was well-tolerated; NOAEL was 4807 mg/kg bw/day in males and 5366 mg/kg bw/day in females; No adverse effects were reported.	Szabo <i>et al.</i> (2012)

F= Female; HLAf = High-lipid algalin flour; M= Male; NOAEL= No-observed-adverse-effect level; ppm = parts *per* million; #= number; WAP = Whole Algal Protein

6.4. Other Studies

6.4.1. Chlordecone detoxification in rodents

The roles that three different algal strains (*C. protothecoides* 902,⁶⁰ *P. zopfii* 822,⁶¹ and *C. vulgaris* 1206⁶²) could play in the detoxification of chlordecone-poisoned rats were explored and compared by Pore *et al.* (1984) in a series of inter-related studies. In previous work (Conte and Pore, 1973; Pore, 1984), both viable cells and cell walls of *C. protothecoides* 902 had been

⁶⁰ 902 *Chlorella protothecoides* BTR (Biotech Research, Inc.) 902; reported depository entries includes ATCC 75667

⁶¹ *Prototheca zopfii* Krüger (Cooke 1962/62-344); aka *P. stagnora* 62-344, and *P. moriformis*; depository designations include ATCC 16527 and UTEX 1442; ATCC Biosafety Level 1 is required for this organism.

⁶² No additional strain information is available.

shown to have a high binding affinity for chlordecone, similar to that of cholestyramine.⁶³ All animals in the current study were female SD rats (175-199 g; 43-51 days of age). All rats received 2.8-5.2 μCi of ^{14}C chlordecone⁶⁴ plus ~ 0.05 mg unlabeled chlordecone in corn oil *via* intraperitoneal (*i.p.*) injection on Day 0. For the initial ingestion study, a subset of the rats ($n = 6$) received a diet supplemented with 4 g/day (20-23 g/kg bw/day) of freeze-dried *C. protothecoides* 902 starting on Day 4 and continuing through Day 17; the corresponding control group ($n = 6$) received the control diet without algal supplementation. As determined by scintillation counting of feces (the primary means of chlordecone excretion) collected on Days 4-13, the half-life ($t_{1/2}$) of chlordecone in the rat was 19.0 days for the *C. protothecoides*-treated group and 35.5 days for the control animals. All rats in the initial study were sacrificed on Day 17. In confirmation of the feces-based findings, the residual ^{14}C -chlordecone radioactivity of abdominal adipose samples⁶⁵ from *C. protothecoides*-treated rats was significantly lower than radioactivity in the control group ($P < 0.01$) (Pore, 1984).

In a series of shorter (*i.e.*, 4-day) studies (Pore, 1984), the $t_{1/2}$ of chlordecone in the rat (as determined by radioactivity in the feces) was 18.7 days for the *C. protothecoides*-treated group ($n = 6$), significantly less than the 40.3 days for the control group ($n = 6$) ($P < 0.01$). When *C. protothecoides* cell walls (isolated by hydrolysis in sulfuric acid from 4 g/day equivalent of *C. protothecoides* 902) were fed to chlordecone-treated rats ($n = 6$), the $t_{1/2}$ was 19.4 days, comparable to the freeze-dried intact cells. In comparison, neither administration (4 g/day) of intact *Prototheca zopfii* nor of intact *C. vulgaris* exhibited a similar effect on the chlordecone $t_{1/2}$ in the rat; measured half-lives (based on radioactivity of feces) were 32.7 days for *C. vulgaris* and 29.5 days for *P. zopfii*. The authors proposed the difference in chlordecone $t_{1/2}$ was due to the presence of relatively large amounts of sporopollenin⁶⁶ (2% of cell dry weight) in the outer trilaminar layer of *C. protothecoides* cell walls. No sporopollenin is present in the cell walls of *C. vulgaris*; intermediate amounts are present in *P. zopfii* cell walls. The authors did not report any adverse effects in the rats that were associated with consumption of viable *C. protothecoides* 902 cells or isolated cell walls.

In summary, exposure of chlordecone-treated rats to either living cells or the cell walls of *C. protothecoides* 902 (20-23 g/kg bw/day) resulted in a significantly decreased chlordecone $t_{1/2}$ compared to the control group. An intermediate effect was observed when intact *P. zopfii* was consumed, but no effect occurred when rats ate intact *C. vulgaris*. The authors believed the reduction of ^{14}C -chlordecone was due to the high amounts of sporopollenin ($\sim 2\%$ dry cell weight) in the *C. protothecoides* cell walls. *C. vulgaris* cell walls contained no sporopollenin and *P. zopfii* contained an intermediate amount. The authors did not report any adverse effects in the

⁶³ Cholestyramine is an ion exchange resin and the primary material used to treat cases of chlordecone intoxication. Cholestyramine binds to chlordecone in the intestine (during primary poisoning or later when absorbed chlordecone is secreted back into the intestine) which prevents reabsorption and allows for elimination with the feces. Without assistance, chlordecone is neither detoxified by the body nor is it effectively eliminated.

⁶⁴ 10 mCi/mmol ^{14}C chlordecone

⁶⁵ Retro-kidney and mesentary

⁶⁶ Rare but natural oxidative carotenoid polymers that occur in a few microorganisms and plants

rats associated with consumption of the living cells or the cell walls of the algae, including *P. zopfii*, at levels up to 4 g/day for the living organisms. This study is summarized in Table 12.

6.4.2. Corroborative pathogenicity study

As corroborative evidence supporting the BSL 1 designation (Section 2.1.) for the *C. protothecoides* S106 source organism utilized in the production of WAP, a pathogenicity study designed to monitor the possible survival or propagation of Microbial Pest Control Agents (MPCA) was conducted using the rat model (Solazyme Roquette Nutritionals LLC, 2012a). The in-life dosing and tissue collection portion of the study was based on US FDA Toxicological Principles for the Safety Assessment of Food Ingredients, Redbook 2000, IV.C. 3a: *Short-Term Toxicity Studies with Rodents* (2003) and US EPA Health Effects Test Guidelines, OPPTS⁶⁷ 885.3050, Acute Oral Toxicity/Pathogenicity (1996); and conducted under GLP.⁶⁸ The tissue culture and analysis portion of the MPCA study was also conducted under GLP⁶⁹ in accordance with the EPA Microbial Pesticide Test Guidelines, OPPTS 885.3050, “Acute Oral Toxicity/Pathogenicity”, US Environmental Protection Agency, Prevention, Pesticides and Toxic Substances (7101). EPA 712-C-96-315, February 1996.

In order to evaluate whether or not *C. protothecoides* S106 microalgae could survive or propagate in the rat, 32 HSD:SD[®] rats (12 weeks old; mean weight \pm 20% for each sex) were randomly assigned to either the treatment ($n = 12$ /sex) or vehicle control ($n = 4$ /sex) group. Either live microalgal cells ($\sim 2.5 \times 10^8$ CFU⁷⁰/rat) in 2 ml vehicle (Defined EBO2⁷¹ minus Co²⁺ and nitrogen) or 2 ml vehicle alone was administered *via* oral gavage to each rat as an acute dose. All treatment animals (24 rats) and half of the vehicle control animals (2 males, 2 females) were housed in one room. The remaining vehicle control animals (2 males, 2 females) were housed in a separate room. All rats were provided 2016CM Harlan Teklad Global Rodent Diet[®] (Harlan Teklad, Inc., Indianapolis, IN) and filtered tap water *ad libitum*. All rats were monitored for viability twice *per* day with cage-side observations twice the first day and once *per* day thereafter. Body weights for control rats were recorded during acclimation and on Days 1, 3, 7, 14 and 21 with terminal weights recorded on Day 22. Body weights for all treatment rats were recorded during acclimation and on Day 1, with terminal weights recorded for the first subgroup ($n = 3$ /sex) on Day 4. Body weights for the second subgroup ($n = 3$ /sex) were recorded on Day 7 with terminal weights recorded on Day 8. The third subgroup body weights ($n = 3$ /sex) were recorded on Days 7 and 14 with terminal weights recorded on Day 15. The fourth subgroup body

⁶⁷ OPPTS = Office of Prevention, Pesticides, and Toxic Substances

⁶⁸ US EPA GLP: Toxic Substances Control Act (TSCA): 40 CFR 792, 1989 and US FDA GLP: 21 CFR 58, 1987

⁶⁹ Chemikaliengesetz (“Chemicals Act”) of the Federal Republic of Germany, Appendix 1 to § 19a as amended and promulgated on June 20, 2002 (BGBl. I Nr. 40 S. 2090), revised October 31, 2006 (BGBl. I Nr. 50 S. 2407); and OECD Principles of Good Laboratory Practice (as revised in 1997), in the OECD Environmental Health and Safety Publications; Series on Principles of Good Laboratory Practice and Compliance Monitoring - Number 1. Environment Directorate, Organisation for Economic Cooperation and Development, Paris 1998

⁷⁰ CFU = Colony Forming Units

⁷¹ Defined EBO2 = A proprietary media formulation developed by Solazyme containing: 3 g/L K₂HPO₄, 5.66 g/L Na₂HPO₄·7H₂O, 1.2 g/L citric acid monohydrate, 1 g/L (NH₄)₂SO₄, 0.23 g/L MgSO₄·7H₂O, 0.03 g/L CaCl₂·2H₂O, 1 mL trace elements minus Co²⁺, 0.225 mL/L antifoam (Sigma 204), 1.5 mL DAS vitamins, glucose to 40 g/L. Defined EBO2 minus Co²⁺ and nitrogen was prepared as above except ammonium sulfate was omitted.

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weights ($n = 3/\text{sex}$) were recorded on Days 7, 14 and 21 with terminal weights recorded on Day 22. Tissue,⁷² blood (*i.e.*, EDTA⁷³/citrate buffered blood and plasma), and fecal samples were collected over a period of three weeks post-dosing (Days 3 (feces only), 4, 8, 15, and 22), transferred (under controlled conditions of 2-8 °C) from PSL (Dayton, NJ) to BSL Bioservice (Planegg, Germany) for analysis (Solazyme Roquette Nutritionals LLC, 2012b).

No mortalities occurred during the in-life portion of the study. No effect in clinical signs, mean body weight or mean body weight gain were found in the treatment groups, compared to the control groups. In addition, no differences were identified in any monitored parameter between the control groups (housed with or isolated from the test group). Although two incidental findings (a small-appearing kidney in one control male and one treatment female) were reported at necropsy, no macroscopic differences were observed among the groups.

Tissue, blood, plasma, and fecal samples were analyzed for the presence of *C. protothecoides* cells using a surface spread method with plate counting.⁷⁴ All sample and control plates were prepared in triplicate with each plate being analyzed in duplicate. After incubation (3-8 days at 30±2 °C), all plates prepared from tissue, blood, and feces samples in addition to negative control (basal medium) and positive control plates (basal medium inoculated with 1:100, 1:1000 and 10,000 dilutions of 2-4 day old *C. protothecoides* S106 cultures) were analyzed by plate counting for CFU/plate of the microalgae. Plate counting determined that algal colony counts were below the limits of detection⁷⁵ for all treatment and control tissue, blood (except for unreadable plates from three control animal samples), plasma, and feces samples for all collection days. Of all plates prepared for analysis, one sample from each of three control animals (two female Day 22 heparinized bloods and one male Day 22 brain) could not be read due to overgrowth by molds and red-colony bacteria. Although the analyses of nearly all fecal samples⁷⁶ from all days were also affected by rapid overgrowth of molds, each of the fecal plates could still be analyzed and each reported an algal count below the limit of detection. No presence or growth of *C. protothecoides* S106 was observed on the negative control plates (< 1 CFU/ml negative control inoculum, equivalent to < 1 CFU/plate). The results of the positive controls, expressed as CFU/ml positive control inoculum (equivalent to CFU/plate), were directly comparable to the CFU/mL of the 2-4 day *C. protothecoides* S106 cultures.

In summary, no adverse or toxic effects related to treatment were observed in any study rat and further, no viable count of the *C. protothecoides* S106 was detected in any examined

⁷² Brain, lung, liver, spleen, kidney, and lymph nodes

⁷³ EDTA = Ethylenediaminetetraacetic acid

⁷⁴ Prior to initiation of the pathogenicity study in the rats, the surface spread method was validated for quantification of *C. protothecoides* S106 in the spiked tissues (brain, lung, liver, spleen, kidney and lymph nodes), blood and feces of control animals (Solazyme Roquette Nutritionals LLC, 2012b). The surface spread method is the method of choice for quantification of colony forming units of aerobic microorganisms (USP <61>).

⁷⁵ All limits of detection (LODs) were normalized to the volume (blood) or weights (tissues and feces) of the samples: <5 CFU/ml plasma and heparinized blood; <14 - < 20 CFU/g brain; <15 - <19 CFU/g lung; <14 - <19 CFU/g liver; <14 - <20 CFU/g spleen; <14 - <19 CFU/g kidney; <14 - <18 CFU/g lymph node; <14 - <31 CFU/g feces for 29 of 32 feces samples with the LOD for one sample at < 54 CFU/g, for a second at < 67 CFU/g, and for a third at < 115 CFU/g (all extremely small, highly diluted samples from Day 15).

⁷⁶ All treatment fecal samples from Days 4, 8, 15, and 22; seven out of eight control fecal samples from Day 22.

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tissues or fluids collected during a three-week post-treatment period to the administration of live algal culture. Under the conditions of this study, *C. protothecoides* S106 was not acutely toxic, pathogenic or toxigenic. This study is summarized in Table 12.

Table 12. Other studies in the rodent model

Method	Concentration	Subjects (#/group)	Results	Reference
Detoxification study, 17-d	0 or 4 g/d (0 or 20-23 g/kg bw/d) live <i>C. protothecoides</i> 902 in feed after rats received 2.8-5.2 µCi of ¹⁴ C chlordecone plus ~0.05 mg unlabeled chlordecone in corn oil via i.p. injection (Day 0)	Female SD rats (6/group)	Chlordecone t _{1/2} in rats fed live 902 was significantly reduced (18.7-19.0 d), compared to the control group (35.5 d) (<i>P</i> < 0.01)	Pore <i>et al.</i> (1984)
Detoxification studies, 4-d	Live <i>P. zopfii</i> 822, live <i>C. vulgaris</i> 1206 or cell walls of <i>C. protothecoides</i> 902 in feed after rats received 2.8-5.2 µCi of ¹⁴ C chlordecone plus ~0.05 mg unlabeled chlordecone in corn oil via I.P. injection (Day 0)	Female SD rats (6/group)	Chlordecone t _{1/2} in rats fed 902 cell walls (19.4 d) was comparable to t _{1/2} in live-902 group; Chlordecone t _{1/2} in rats fed live 822 or live 1206 were 29.5 and 32.7 d, respectively, and did not differ from the control. The authors did not report any adverse effects to consumption of live cells or cell walls.	Pore <i>et al.</i> (1984)
Pathogenicity study, 21-d	0 or ≥ 2.5x10 ⁸ CFU live <i>C. protothecoides</i> S106 /rat in vehicle (Defined EB02 minus Co ²⁺ and nitrogen) via oral gavage (Day 0); Treatment rats (3M,3F/ time point) sacrificed on Days 4, 8, 15 and 22; Control rats sacrificed on Day 22	Hsd:SD [®] rats (12M/12F treatment group; 4M/4F vehicle control group)	No mortalities; No effect on clinical signs, bw, bw gain or macroscopic examination at necropsy; Algal counts below the limit of detection in tissue, plasma, heparinized blood, and fecal samples; <i>C. protothecoides</i> S106 was not acutely toxic or pathogenic	Solazyme, (2012a)

CFU = Colony Forming Units; Defined EBO = A proprietary media formulation developed by Solazyme containing: 3 g/L K₂HPO₄, 5.66 g/L Na₂HPO₄·7H₂O, 1.2 g/L citric acid monohydrate, 1 g/L (NH₄)₂SO₄, 0.23 g/L MgSO₄·7H₂O, 0.03 g/L CaCl₂·2H₂O, 1 mL trace elements minus Co²⁺, 0.225 ml/L antifoam (Sigma 204), 1.5 ml DAS vitamins, glucose to 40 g/L. Defined EB02 minus Co²⁺ and nitrogen was prepared as above except ammonium sulfate was omitted; bw = body weight; d = days; F = female; I.P.= Intraperitoneal; M = Male; t_{1/2} = half life

6.5. Genotoxicity

6.5.1. Mutagenicity assay for WAP

The mutagenic potential of WAP⁵⁶ was evaluated by bacterial reverse mutation assay (*i.e.*, Ames test) using standard plate incorporation (Experiment I) and pre-incubation

(Experiment II) methods with and without S9 metabolic activation⁷⁷ (Szabo *et al.*, 2013). Tester strains included *Salmonella typhimurium* TA100 and TA1535 and *Escherichia coli* WP2uvrA for detection of base-pair substitutions and *S. typhimurium* TA98 and TA1537 for detection of frame-shift mutations. The assays were performed in compliance with international guidelines⁷⁸ and conducted under GLP conditions.⁶⁹ In the pre-experiments using test strains TA 98 and TA 100, 5000 µg/plate WAP was selected as the highest dose for all test strains and conditions due to a lack of cytotoxicity. In Experiment I, six dose levels (5000, 2500, 1000, 316, 100 and 31.6 µg/plate) were prepared and tested; in Experiment II, seven dose levels (1000, 316, 100, 31.6, 10.0 and 3.16 µg/plate) were prepared. In the absence of activation, sodium azide (NaN₃) served as positive control for *S. typhimurium* TA100 and TA1535, 4-nitro-*o*-phenylene-diamine (4-NOPD) for *S. typhimurium* TA98 and TA1537 and methylmethanesulfonate (MMS) for *E. coli* WP2uvrA. In the presence of activation, the positive control for all bacterial strains was 2-aminoanthracene (2-AA). The negative and solvent control for all tests was distilled water.

WAP was observed to precipitate at concentrations of 1000 µg/plate and higher in all test strains in the presence and absence of S9 mix in Experiments I and II. Cytotoxic effects of the test substance were observed in test strain TA 1537 at the two highest concentrations (2500 and 5000 µg/plate) in Experiment I with metabolic activation and at 2500 µg/plate, the highest concentration tested, in Experiment II without metabolic activation. Although a reduction in the number of revertants in test strain TA 1537 at a concentration of 316 µg/plate in Experiment II (without activation) met the criteria for cytotoxicity, no dose-response relationship was in evidence and the effect was determined not to be biologically relevant. Regardless of the presence or absence of metabolic activation, dose-dependent increases in the number of revertant colonies of greater than twice the negative control values were not observed in any strain treated with WAP in either experiment. Under the conditions of this study, WAP did not cause gene mutations by base pair changes or frame shifts in the genomes of the tester strains and was, therefore, not a mutagen.

6.5.2. Mutagenicity assay for HLA F

The mutagenic potential of the similar HLA F material was evaluated by bacterial reverse mutation assay (Szabo *et al.*, 2012; FDA, 2013) using standard plate incorporation (Experiment I) and pre-incubation (Experiment II) methods with and without S9 metabolic activation⁷⁹ under the same conditions^{69,78} as those previously described for the testing of WAP. Based on pre-experiments, 5000 µg/plate HLA F was selected as the highest dose for all test strains and conditions, except for TA 1537 in Experiment II without activation, where 1000 µg/plate was selected as the highest dose due to cytotoxic effects of the test substance. For the 5000 µg/plate series, five lower dose levels (2500, 1000, 316, 100 and 31.6 µg/plate) were also prepared and

⁷⁷ S9 metabolic mix = The supernatant fraction of rat liver homogenate (derived from male Wistar and SD rats pretreated with the inducers phenobarbital and β-naphthoflavone) mixed with Cofactor-I.

⁷⁸ Ninth Addendum to OECD Guidelines for Testing of Chemicals, Section 4, No. 471, "Bacterial Reverse Mutation Test", adopted July 21, 1997 and Commission Regulation (EC) No. 440/2008 B.13/14, "Mutagenicity – Reverse Mutation Test using Bacteria", dated May 30, 2008.

⁷⁹ Similar to the S9 metabolic mix used to test WAP, except that the supernatant fraction of rat liver homogenate was derived only from male Wistar rats.

tested; for the 1000 µg/plate series, six lower dose levels (316, 100, 31.6, 10.0, 3.16 and 1.00 µg/plate) were also prepared. In the absence of activation, NaN₃ served as positive control for *S. typhimurium* TA100 and TA1535, 4-NOPD for *S. typhimurium* TA98 and TA1537 and MMS for *E. coli* WP2uvrA. In the presence of activation, the positive control for all bacterial strains was 2-AA. The negative control for all tests was distilled water and the solvent control was dimethyl sulfoxide (DMSO).

Precipitation of HLAf was visually observed in all test strains at concentrations of 2500 µg/plate and higher in Experiment I and II in the absence of S9 mix and in all test strains at a concentration of 5000 µg/plate in Experiment I and II in the presence of S9. In Experiment I cytotoxic effects of the test substance were observed in test strain TA 1537 at concentrations of 316 µg/plate and higher without metabolic activation and at concentrations of 2500 µg/plate with metabolic activation. In Experiment II these same effects were observed in test strains TA 98 and TA 100 at concentrations of 316 µg/plate and higher without metabolic activation and in test strain TA 1537 at concentrations of 10.0 µg/plate and higher without metabolic activation and concentrations of 316 µg/plate and higher with metabolic activation. Regardless of the presence or absence of metabolic activation, dose-dependent increases in the number of revertant colonies of greater than twice the negative control values were not observed in any strain treated with HLAf in either experiment. Under the conditions of this study, HLAf was not a mutagen.

6.5.3. Clastogenicity assay for WAP

The clastogenic potential of WAP⁵⁶ was evaluated using the *in vivo* bone marrow chromosome aberration assay in the mouse (Szabo *et al.*, 2013). The assays were performed under GLP conditions⁶⁹ and in compliance with international guidelines.⁸⁰ Prior to the main experiment, a range-finding study (OECD guideline 475) was used to determine the maximum tolerated dose (MTD) of WAP. Due to a lack of observed toxicity, the MTD defaulted to 2000 mg/kg bw, the highest dose evaluated for the assay (in accordance with OECD guideline 475). This dose was then selected as the maximum in the main study. Prepared in physiological saline one hour before treatment, WAP at a dose of 2000 mg/kg bw was administered *via* oral gavage to ten male and ten female NMRI mice (7 – 13 weeks old) in a single application at study initiation. Negative control animals (*n* = 10/sex) were administered the vehicle in similar volumes (10 ml/kg bw). Positive control mice (*n* = 5/sex) received 40 mg/kg bw cyclophosphamide (CPA) in physiological saline *via i.p.* injection. Bone marrow cells were harvested 24 and 48 h later. Exposure times were 24 hours and 48 hours post-administration for the treatment and negative control groups (*n* = 5/sex/group/time) and 24 hours post-administration for the positive control group. After bone marrow cells were fixed and stained, 100 metaphases⁸¹ were scored for cytogenic damage to determine the incidence of structural chromosomal aberrations (*i.e.*, breaks, fragments, deletion exchanges, chromosomal

⁸⁰ The Ninth Addendum to OECD Guidelines for Testing of Chemicals, Section 4, No. 475, “Mammalian Bone Marrow Chromosome Aberration Test”, adopted July 21, 1997 and Commission Regulation (EC) No. 440/2008 B.11, “Mutagenicity – Mammalian Bone Marrow Chromosome Aberration Test”, dated May 30, 2008.

⁸¹ For animals that showed a distinct positive result in fewer than 100 metaphases, 50 metaphases were examined (in accordance with OECD Guideline 475).

disintegrations and gaps). In addition, a minimum of 1000 cells were evaluated for cytotoxicity to determine the mitotic index (*percent* of cells in mitosis).

The mean values of aberrant cells in the 24- and 48-hour negative control and test groups (Table 13) remained within the range of the historic negative controls (0 – 5% in male and 0 – 3% in female mice from years 2001–2010). No dose-dependent, biologically relevant or statistically significant increase in the incidence of aberrant cells was observed in any 24-hour or 48-hour dose group compared to its corresponding negative control group. The mitotic index values for the 24-hour male and female test groups and 48-hour female test group remained in the range of the corresponding negative control (Table 13); no statistically significant change to the mean mitotic index occurred in these groups. For the 48-hour male test group, however, the mean mitotic index value (Table 13) was significantly lower than the value for the corresponding negative control ($P < 0.01$). Because the decrease was determined to be an effect of biological variability among the animals, it was not considered biologically relevant. The validity of the chromosome aberration assay was verified by (1) the weight variation of the mice not exceeding $\pm 20\%$ of the pre-dose mean weight of each sex, (2) the lack of biologically significant increases in aberrant cell values in the negative control group, and (3) the biologically significant induction of aberrant cells in the positive control group ($P < 0.01$). Under the conditions of this study, WAP did not induce cytotoxicity or structural chromosome aberrations and was therefore, not clastogenic.

Table 13. Summary of chromosome aberration assay results for WAP

Study Groups (<i>n</i> = 5)	Metaphases	Aberrant Cells (Total/ % \pm SD)	Mitotic Index
Negative Control, 24 h			
Male	500	3/ 0.6 \pm 0.9	6.22
Female	500	3/ 0.6 \pm 0.5	6.60
Positive Control, 24 h			
Male	350 ^a	138/ 39.4 \pm 12.3 [*]	5.02
Female	300 ^b	94/ 31.3 \pm 4.9 [*]	3.36
Treatment Group, 24 h			
Male	500	4/ 0.8 \pm 0.4	8.08
Female	500	2/ 0.4 \pm 0.5	8.26
Negative Control, 48 h			
Male	500	1/ 0.2 \pm 1.6	9.36
Female	500	3/ 0.6 \pm 0.5	8.74
Treatment Group, 48 h			
Male	500	3/ 0.6 \pm 0.5	4.60 [*]
Female	500	1/ 0.2 \pm 0.4	9.50

6.5.4. Clastogenicity assay for HLA F

The clastogenic potential of the similar HLA F material was evaluated in an *in vivo* bone marrow chromosome aberration test in the mouse (Szabo *et al.*, 2012; FDA, 2013) under the same conditions as those previously described for the testing of WAP. Prior to the main experiment, the MTD for the range-finding study defaulted, due to a lack of observed toxicity, to

2000 mg/kg bw, the highest dose evaluated for the assay. This dose was then selected as the maximum in the main study. Prepared in cottonseed oil one hour before treatment, HLAF at a dose of 2000 mg/kg bw was administered *via* gavage to ten male and ten female NMRI mice (7-13 weeks old) in a single application at study initiation. Negative control mice ($n = 10/\text{sex}$) were given the vehicle in similar volumes. Positive control animals ($n = 5/\text{sex}$) received a single dose of 40 mg/kg bw of CPA in physiological saline *via i.p.* injection. Bone marrow cells were harvested 24 hours and 48 hours post-administration for the treatment and negative control groups ($n = 5/\text{sex}/\text{group}/\text{time}$) and 24 hours post-administration for the positive control group. After bone marrow cells were fixed and stained, 100 metaphases⁸¹ were scored for cytogenic damage to determine the incidence of structural chromosomal aberrations. In addition, approximately 1000 cells were evaluated for cytotoxicity to determine the mitotic index. Although the male and female positive controls exhibited biologically and statistically significant increases in aberrant cell values ($P < 0.01$ for both *vs.* corresponding negative control), no dose-dependent, biologically relevant or statistically significant increase in the incidence of aberrant cells or in the mitotic index was observed in the 24-hour or 48-hour dose groups compared to their corresponding negative controls. The findings (aberrant cell values and mitotic indices) for all groups except for those of the positive control were in the range of the historic negative controls. Under the conditions of this study, HLAF was not clastogenic.

In summary, neither WAP nor HLAF exhibited mutagenicity under the conditions of the bacterial reverse mutation assay or clastogenicity under the conditions of the *in vivo* bone marrow chromosome aberration assay. The genotoxicity studies are summarized in Table 14.

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Table 14. Genotoxicity studies

Assay	Test system	Concentrations	Results	Reference
Bacterial reverse mutation assay	<i>S. typhimurium</i> TA98, TA100, TA1535, and TA1537; and <i>E. coli</i> WP2uvrA	Experiment I (standard plate incorporation): Up to 5000 µg/plate WAP in distilled water for all test strains and conditions (with and without S9 activation); Experiment II (pre-incubation): Up to 2500 µg/plate WAP in distilled water for all test strains and conditions.	Not mutagenic	Szabo <i>et al.</i> (2013)
Bacterial reverse mutation assay	<i>S. typhimurium</i> TA98, TA100, TA1535, and TA1537; and <i>E. coli</i> WP2uvrA	Experiment I (standard plate incorporation): Up to 5000 µg/plate HLAF in DMSO for all test strains and conditions (with and without S9 activation); Experiment II (pre-incubation): Up to 5000 µg/plate HLAF in DMSO for all test strains and conditions except for TA 1537 without S9 activation where 1000 µg/plate was highest dose due to cytotoxic effects.	Not mutagenic	Szabo <i>et al.</i> (2012)
<i>In vivo</i> bone marrow chromosome aberration assay	NMRI Mouse (10M/10F/group)	0 and 2000 mg/kg WAP in physiological saline <i>via</i> gavage; single dose	Not clastogenic	Szabo <i>et al.</i> (2013)
<i>In vivo</i> bone marrow chromosome aberration assay	NMRI Mouse (10M/10F/group)	0 and 2000 mg/kg bw HLAF in cottonseed oil <i>via</i> gavage; single dose	Not clastogenic	Szabo <i>et al.</i> (2012)

DMSO = Dimethyl sulfoxide; HLAF = High-lipid algalin flour; WAP = Whole Algal Protein

6.6. Carcinogenesis

No studies addressing carcinogenicity were discovered in the scientific literature for *C. protothecoides* or *A. protothecoides*.

6.7. Observations in Humans

Not intended to be consumed as a stand-alone food (and therefore, similar to flour, salt or cooking oil), WAP is a pale yellow to green ingredient³ that has a slight savory flavor. When used at its intended levels in food, taste of the final food is not adversely affected.

6.7.1. Allergenic Potential

A search of the scientific literature did not indicate any association of allergy or allergic response to *C. protothecoides* or to any *Auxenochlorella* or *Prototheca* species. Published studies

of allergy instead have primarily been limited to *Chlorella* species⁸² such as *C. vulgaris*, *C. pyrenoidosa*, *C. saccharophila* and *C. homosphaera* (Tiberg *et al.*, 1990a; Tiberg *et al.*, 1990b; Genitsaris *et al.*, 2011). Although allergy to *Chlorella* has generally presented as hypersensitization resulting from exposure to algae-infested waters or respiratory allergy to airborne⁸³ algae (Bernstein and Safferman, 1966; Bernstein and Safferman, 1973; Tiberg *et al.*, 1995; Chrisostomou *et al.*, 2009; Genitsaris *et al.*, 2011), there is a published case study of oral allergy in an 11-year-old boy (Yim *et al.*, 2007). Three months into a daily regimen of *Chlorella* food supplements (200 mg/tablet, 10 tablets/day), acute tubulointerstitial nephritis⁸⁴ was diagnosed in the child. Renal function improved with cessation of the *Chlorella*⁸⁵ supplements and a six-month regimen of corticosteroids. A follow-up skin prick test (100 mg and 200 mg *Chlorella* in 5 ml distilled water) six months after steroid therapy failed to produce positive wheal reactions for the 100 mg or 200 mg test amounts. In a previous study by Tiberg and Einarsson (Tiberg and Einarsson, 1989), the allergenic potencies of eight strains within four species of *Chlorella* (*C. vulgaris*, *C. homosphaera*, *C. saccharophila*, and *C. fusca*) were compared in an attempt to define a representative extract preparation for the genus. Due to the highly variable allergenic activity of the eight strains, the authors concluded that development of a representative extract preparation for the genus was unlikely and that “selection of a strain is a crucial step for the production of an allergen extract from *Chlorella*.”

6.7.1.1. Human repeat-insult patch test with WAP

The potential for induced allergenic response to WAP⁵⁶ when consumed as a food was investigated *via* dermal sensitization of human subjects (Szabo *et al.*, 2013). The study design was in accordance with the applicable guidelines for the protection of human subjects for research as outlined in 21 CFR §50 and in accordance with the accepted standards for Good Clinical Practices (GCPs), International Conference on Harmonization (ICH Expert Working Group, 1996) and the standard practices of Thomas J. Stephens and Associates, Inc. (Carrollton, TX). Performance of a dermal patch test in humans as a means of predicting food allergy by the occurrence of contact dermatitis is a noninvasive approach that holds low risk for the safety of the participants. Written informed consent complying with 21 CFR §50.25 was obtained from each subject prior to study enrollment. A total of 130 healthy subjects enrolled in the Human Repeat Insult Patch test (HRIPT) for WAP; 111 participants⁸⁶ (28 men, 83 women; average subject age of 45.06 ± 12.97 years) completed the study.

⁸² When considering species identified in articles published prior to 2000, the possibility of reclassification is a concern especially for *C. vulgaris* and *C. pyrenoidosa* species. Verification against the culture collections of the depositories is recommended.

⁸³ Aerosolized or associated with house dust.

⁸⁴ Tubulointerstitial nephritis is a common cause of acute renal failure from immune-mediated tubulointerstitial injury; hypersensitivity reactions can induce allergic response in the renal interstitium (Yim *et al.*, 2007).

⁸⁵ Composition of the tablets was not defined beyond the genus.

⁸⁶ The 111 subjects completing the study identified their ethnicity/race as follows: 11 African Americans (9.9%), 1 Asian (0.9%), 77 Caucasians (69.4%), 13 Hispanics (11.7%), 2 Native Americans (1.8%), 1 Pacific Islander (0.9%), and 6 subjects of mixed ethnicity (5.4%).

The HRIPT developed by Rizer and Nozawa (Trookman *et al.*, 2011) consisted of two phases, an induction phase and a challenge phase. The three-week induction phase included nine patch periods. During each period, sufficient WAP test material to cover the test site (approximately 2 cm² in area) was applied under a patch to healthy skin on the upper back of each subject. The patch was removed 48 hours after application or about two hours before a study visit. Induction sites were graded 48 to 72 hours after each application using a standardized skin irritation scale (Berger and Bowman, 1982). A minimum of 12 days after application of the last induction patch, challenge patches were applied to the original test site and to a naïve site, also on the upper back. These were removed 48 hours after application or about two hours before the study visit. Challenge sites were graded 48 hours and 96 hours post-application. Occlusive patches (non-woven cotton pads covered by Blenderm tape and held securely to skin on all sides with a porous, hypoallergenic tape) were used for the first two patch periods in the induction phase; semi-occlusive patches (non-woven cotton pads covered and held securely to skin on all sides with a porous, hypoallergenic tape) were used for all later patch periods in the induction phase and for the entire challenge phase.

Of the 130 subjects who enrolled, one was discontinued for noncompliance, 16 for missing more than one study visit, and one withdrew for a cardiac condition unrelated to the test material. One additional subject withdrew after grading of the first occlusive patch determined moderate site reaction to the occluded test material. The site scored a “1” for erythema with evident papules. The subject withdrew from the study and the reaction was followed to resolution.

Because increasing instances of site irritation were broadly observed among the study participants at the second grading (23%; Table 16), semi-occlusive patches were substituted for occlusive patches for the remainder of the study. At the ninth (final) grading period in the induction phase, the test sites of three participants (2.7%) exhibited a mild erythema⁸⁷ (faint to definite pink) while the test sites of the other 108 participants (97.3%) presented with no visible erythema (Table 16). Changing the patch system from occlusive to semi-occlusive resolved the cumulative irritation evident in the early stages of the induction phase. The results of the challenge phase further demonstrated that the test material did not induce an allergic response in the study group. At the 96-h grading in the challenge phase, the original application site of one subject (0.9%) and naïve sites of two subjects (1.8%) exhibited mild erythema (Table 16). No other subjects demonstrated irritation.

Regarding the one subject withdrawn during the occlusive portion of the test (after the first patch period), the total evidence suggests that this individual is more likely to have experienced irritation, rather than allergic response. Although pre-existing skin sensitization is suggested by the rapidity (first of nine 48-h patch periods) and severity (papules) of the response, the response was not confirmed (*via* oral food challenge) to result from a true allergic reaction. Because erythema is known to result from local irritation (Cudowska and Kaczmarek, 2005), gradings of “1”, mild erythema without infiltration, are usually regarded as negative and a

⁸⁷ Because redness can result from local irritation, mild erythema alone is not treated as potentially indicative of allergic response.

positive reaction is assessed only if erythema with infiltration or papules is present. Distinguishing irritation from allergy can, however, be challenging for test materials with inherent irritant potential (McNamee *et al.*, 2008).⁸⁸ In addition, it is widely known that repeated exposure to a dermal irritant can result in a cumulative irritation reaction (Trookman *et al.*, 2011) and that the use of occlusive patching (an exaggerated exposure condition frequently used for this very reason), can exacerbate irritant effects (McNamee *et al.*, 2008). In this study, WAP was applied as a dry solid to subjects' backs under occlusive patching without dilution or extraction.⁸⁹ At the end of the second patch period (occlusive) nearly one-fourth (23%) of study volunteers exhibited some degree of patch site irritation; 23 participants presented with mild erythema (gradings of "1"), two individuals presented with mild erythema and one additional subject presented with papules only (no erythema). The use of semi-occlusive patching during the remainder of the study was found to resolve nearly all irritation concerns in the 111 remaining subjects for the remaining weeks of the study (Table 15). At the end of the induction phase, only three subjects presented with mild erythema at the application site. At the end of the challenge phase, one presented with mild erythema at the original site and two at the alternative (naïve) site. As an additional consideration, while atopic patch tests (APTs) have been shown to generate negative predictive values of about 90%⁹⁰ and have been found particularly useful in predicting late phase (T-cell-mediated) reactions⁹¹ (Cudowska and Kaczmarek, 2005; Chung *et al.*, 2010), positive predictive values have been substantially lower (50% and 57% compared to DBPCFC⁹² for milk and egg allergy, respectively, (Chung *et al.*, 2010); 29% for milk, casein, egg white and yolk (Peron *et al.*, 2011). A tendency for false positives (20% compared to DBPCFC for milk allergy in older children (Cudowska and Kaczmarek, 2005)) is a recognized limiting factor of APT.

In summary, although mild-moderate irritation was identified in several subjects, WAP did not, under the conditions of this test, induce contact sensitization (allergic contact dermatitis) in any subject completing the study which suggests a low likelihood of food allergy.

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⁸⁸ Wheat gluten is an example of a test material suspected of inducing false positive (irritant) reactions (Turjanmaa *et al.*, 2006; McNamee *et al.*, 2008).

⁸⁹ When a test material is a potential irritant, dilution or extraction is recommended as a means of decreasing the incidence of false positive responses (Turjanmaa *et al.*, 2006; McNamee *et al.*, 2008).

⁹⁰ 95.9% and 88.9% compared to double-blind placebo-controlled food challenge (DBPCFC) for milk and egg allergy, respectively (Chung *et al.*, 2010); and 90% for milk, casein, egg white and yolk (Peron *et al.*, 2011).

⁹¹ In comparison, skin prick tests (SPT) and food-specific IgE antibodies *via* serological assay are indicative only of early (IgE-mediated) food allergy reaction (Cudowska and Kaczmarek, 2005).

⁹² DBPCFC = Double-Blind Placebo-Controlled Food Challenge.

Table 15. Score frequencies for WAP at each grading during the HRIPT induction and challenge phases.

Score	Induction Phase								Challenge Phase				
	G1	G2	G3	G4	G5	G6	G7	G8	G9	48 O	96 O	48 A	96 A
0	97	85	105	109	106	110	106	107	108	107	110	104	109
0P	0	1	1	0	1	0	0	0	0	0	0	0	0
1	13	23	4	1	4	1	5	4	3	4	1	7	2
1P	1	2	1	1	0	0	0	0	0	0	0	0	0
Total	111	111	111	111	111	111	111	111	111	111	111	111	111

0 = No visible erythema; 1 = Mild erythema (faint pink to definite pink); 48 = 48-hour observation; 96 = 96-hour observation; A = Alternate (naïve) site; G = Grading; HRIP = Human repeat-insult patch; O = Original site; P = papules; WAP = Whole Algal Protein

6.7.1.1. Human repeat-insult patch test with HLA F

The potential for the induction of an allergic response from HLA F when consumed as a food has been investigated in human subjects (Szabo *et al.*, 2012; FDA, 2013) using the same approach as described previously for WAP. A total of 130 healthy subjects enrolled in the HRIPT for HLA F and 110 participants⁹³ (28 men, 82 women; average subject age of 45.01 ± 13.01 years) completed the study.

Of the 130 subjects who enrolled, one was discontinued for noncompliance, 16 for missing more than one study visit, and one withdrew for a cardiac condition unrelated to the test material. Two additional subjects were withdrawn after grading of the first and second occlusive patches determined moderate site reactions to the test material. One subject was scored during the first grading as having severe erythema (very intense redness) with edema (swelling), bullae⁹⁴ and spreading at the application site; this subject withdrew from the study and the reaction was followed to resolution. The second subject was scored during the first grading as having severe edema with bullae at the application site. For this subject, an alternate site was used for the second patching and both the original and alternate sites were evaluated during the second grading. At the second grading, the original site scored “severe” for erythema with edema and papules⁹⁵ and the alternate site scored as “severe” edema with bullae; the subject was then withdrawn and the reactions were followed to resolution.

Because increasing instances of site irritation were broadly observed among the study participants at the second grading (37%; Table 16), semi-occlusive patches were substituted for occlusive patches for the remainder of the study. At the ninth (final) grading in the induction phase, the test sites of four participants (3.6%) exhibited a mild erythema while the test sites of 106 participants (96.4%) presented with no visible erythema. After the change to semi-occlusive patching, no cumulative irritation was observed in the induction phase. The results of the

⁹³ The 110 subjects completing the study identified their ethnicity/race as follows: 11 African Americans (10.0%), 1 Asian (0.9%), 76 Caucasians (69.1%), 13 Hispanics (11.8%), 2 Native Americans (1.8%), 1 Pacific Islander (0.9%), and 6 subjects of mixed ethnicity (5.5%).

⁹⁴ Vesicles (small, circumscribed elevations having translucent surfaces so that fluid is visible (*i.e.*, blister-like)) with a diameter > 0.5 cm; vesicles may coalesce to form one or a few large blisters that fill the patch site.

⁹⁵ Small red solid elevations; when touched the affected surface has a granular feeling.

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challenge phase demonstrated that the test material did not induce an allergic response in the study group. At the 96-hour grading in the challenge phase, the original application sites of three subjects (2.7%) and naïve site of one subject (0.9%) exhibited mild erythema. No other subjects demonstrated irritation.

Regarding the two subjects withdrawn during the occlusive portion of the test (one after the first and one after the second patch period), the total evidence suggests that these individuals are more likely to have experienced irritation, rather than allergic response. Although pre-existing skin sensitization was strongly suggested by the rapidity (first of nine 48-h patch periods), severity (edema and papules/bullae), and persistence of the responses (3-4 weeks to resolve) in both subjects, neither of the responses was confirmed (*via* oral food challenge) to result from a true allergic response.

In this study, HLAf was applied as a dry solid to subjects' backs under occlusive patching without dilution or extraction.⁹⁶ At the end of the second patch period (occlusive) more than one-third (37%) of study volunteers exhibited some degree of patch site irritation (*i.e.*, erythema) with three individuals also presenting with papules and a fourth with edema. The use of semi-occlusive patching during the remainder of the study was found to resolve nearly all irritation concerns in the 110 remaining subjects for the remaining weeks of the study (Table 16). At the end of the induction phase, only four subjects presented with mild erythema at the application site. At the end of the challenge phase, three presented with mild erythema at the original site and only one at the alternative (naïve) site.

In summary, although mild-moderate irritation was identified in several subjects, HLAf did not, under the conditions of this test, induce contact sensitization (allergic contact dermatitis) in any subject completing the study which suggests a low likelihood of food allergy.

Table 16. Score frequencies for HLAf at each grading during the HRIPT induction and challenge phases.

Score	Induction Phase									Challenge Phase			
	G1	G2	G3	G4	G5	G6	G7	G8	G9	48 O	96 O	48 A	96 A
0	81	68	99	106	98	106	106	105	106	106	107	103	109
0P	0	0	1	0	3	0	0	0	0	0	0	0	0
0R	0	1	0	0	0	0	0	0	0	0	0	0	0
1	27	36	9	3	9	4	4	3	4	4	3	7	1
1P	2	3	1	1	0	0	0	2	0	0	0	0	0
2	0	1	0	0	0	0	0	0	0	0	0	0	0
2E	0	1	0	0	0	0	0	0	0	0	0	0	0
Total	110	110	110	110	110	110	110	110	110	110	110	110	110

0 = No visible erythema; 1 = Mild erythema (faint pink to definite pink); 2 = Moderate erythema (definite redness); 48 = 48-hour observation; 96 = 96-hour observation; A = Alternate (naïve) site; E = Edema; G = Grading; HLAf = High-lipid algalin flour; HRIP = Human repeat-insult patch; O = Original site; P = papules, R = Removal of the patch at other than the assigned time

⁹⁶ When a test material is a potential irritant, dilution or extraction is recommended as a means of decreasing the incidence of false positive responses (Turjanmaa *et al.*, 2006; McNamee *et al.*, 2008).
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6.7.2. Infection

Even though microalgae are ubiquitous organisms, algal infections are rare in humans and other mammals. Of those that do occur, the most common are protothecosis and chlorellosis, pseudofungal diseases caused, respectively, by opportunistic members of the achlorophyllous *Prototheca* spp. and green *Chlorella* spp. (Ramírez-Romero *et al.*, 2010). The two *Prototheca* species known to be disease agents are *P. wickerhamii*, the cause of most human protothecosis and *P. zopfii*, the usual causative agent in animals (Chandler *et al.*, 1978; Jagielski and Lagneau, 2007; Tap *et al.*, 2012). Thus far, the *Chlorella* species responsible for incidents of chlorellosis have not yet been identified. Chlorellosis is usually differentiated from protothecosis by the color of lesions and/or affected organs. Protothecosis lesions tend to be white, ivory, or cream-colored like the achlorophyllous causative organism and are difficult to distinguish in culture from common infectious yeast organisms (*i.e.*, *Candida* and *Cryptococcus*) (Tap *et al.*, 2012). Chlorellosis lesions, on the other hand, are generally bright green or emerald in color (Chandler *et al.*, 1978).

Protothecosis typically manifests in animals and humans as localized (*i.e.*, cutaneous or articular) or disseminated systemic infection⁹⁷ (Tap *et al.*, 2012). In the cow, protothecosis is commonly diagnosed as the cause of bovine mastitis. Although infection in cats and goats is also usually localized and cutaneous, infection in dogs can be severe, involving the eyes, brain, and internal organs (Jagielski and Lagneau, 2007; Satoh *et al.*, 2010). In humans protothecosis most commonly presents as skin infection, usually from contamination of wounds or other breaks in the skin with dirty water (Torres *et al.*, 2003). Individuals who become ill with systemic infection are nearly always debilitated or immunocompromised (Torres *et al.*, 2003; Jagielski and Lagneau, 2007; Ramírez-Romero *et al.*, 2010; Satoh *et al.*, 2010; Tap *et al.*, 2012). On a yearly basis, 2-5 cases of infection are typically reported, with 108 total cases of human protothecosis reported over a 25 year period (Krcmery Jr., 2000).

Chlorellosis, on the other hand, has been reported in a variety of domestic and wild animals, including dogs, cattle, sheep, gazelle, dromedaries and beavers, but in only one human. The single reported incident of human infection presented as localized cutaneous lesions in surgical wounds exposed to river water (Ramírez-Romero *et al.*, 2010); the causative species was not identified. Although infection in the human patient was mild, chlorellosis can cause systemic disease in livestock (Chandler *et al.*, 1978; Ramírez-Romero *et al.*, 2010). Most bovine infections are mild (*i.e.*, infected lymph nodes found during slaughterhouse inspection); chlorellosis in sheep, however, is often enteric and frequently the cause of morbidity and mortality (Chandler *et al.*, 1978; Ramírez-Romero *et al.*, 2010). Although infection usually occurs through environmental contamination of open wounds, infection in animals has also been associated with drinking sewage water or stagnant water, or grazing pasture irrigated with untreated sewage water (Ramírez-Romero *et al.*, 2010).

In summary, a single incident of human chlorellosis and 108 cases of human protothecosis over a 25 year period have been reported. The chlorellosis infection appeared in surgical wounds exposed to contaminated water and was limited to cutaneous lesions. Because

⁹⁷ The infection of internal organs that often results in altered immune response.
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lesional organisms are rarely cultured for genotyping, no evidence is available to indicate which *Chlorella* strain was the opportunistic pathogen in the one human patient or in the animals that are occasionally infected. Considering that only one case of chlorellosis in humans has been reported compared to 108 reported cases of human protothecosis, it is highly unlikely that *C. protothecoides* has or will be the cause of human disease.

6.7.3. Photosensitive dermatitis

In 1977, at least 23 residents of Tokyo were diagnosed with photosensitive dermatitis⁹⁸ (Tamura *et al.*, 1979; Jassby, 1988). Investigation revealed that each patient had consumed the same brand of *Chlorella* tablet (“Kenbi *Chlorella*”) and, further, that the tablets contained PheideA and its ester in high quantities (up to 8.2 mg/g total PheideA; (Jassby, 1988). A subsequent feeding trial in mice confirmed the association when administration of the suspect tablets induced the phototoxic response and administration of *Chlorella* tablets having lower concentrations of Pheides (< 10% of suspect concentrations) did not. The researchers were also able to establish a linear dose-response relationship between inflammation severity and the Pheide content of the administered tablets (Jitsukawa *et al.*, 1984; Jassby, 1988).

Pheophorbide-associated phototoxicity occurs after ingested Pheides enter the circulatory system and distribute throughout the body. When Pheides that have deposited in the skin or other near-surface tissues are exposed to light, oxygen is generated and the fatty acids of nearby cell membranes are subsequently oxidized which leads to cell rupture, damage to dermal capillaries and escalating inflammation (Jitsukawa *et al.*, 1984; Jassby, 1988). In the Tokyo incident, a change in the manufacturing process (the use of ethanol during granule formation of the dried *Chlorella* prior to pelleting) was determined to have been the cause of the high PheideA concentrations (Jitsukawa *et al.*, 1984). The concentrations of existing and potential total PheideA permitted in algae preparations were limited, respectively, to 0.8 mg/g and < 1.2 mg/g by the Japanese Public Health Ministry in 1981 (Jassby, 1988).

Because Pheides are natural degradation products of chlorophylls (Chls), they are present in all photosynthetic plants. In nature, Chl catabolites, including the phototoxic Pheides, typically increase in concentration during leaf senescence, fruit ripening or other incidents of “de-greening” (Jassby, 1988; Hörtensteiner *et al.*, 2000). During “de-greening” events, Pheides form from Chl through the step-wise loss of the phytol residue *via* chlorophyllase followed by the loss of the central magnesium atom *via* magnesium dechelatase (Hörtensteiner, 1999; Hörtensteiner *et al.*, 2000). As mentioned previously, certain manufacturing steps can affect Pheide formation in Chl-containing microalgae products including the use of ethanol, acetone or methanol during processing which can enhance chlorophyllase activity and production of Pheides. On the other hand, heating Chl-containing materials to 100 °C for three minutes is sufficient to inactivate the chlorophyllase enzyme (Jassby, 1988). To ensure against Pheide formation in preparations of microalgae that contain Chl, enzyme inactivation is often combined with an avoidance of processing steps using ethanol, acetone or methanol (Jassby, 1988). The WAP manufacturing process includes a pasteurization step early in the recovery process that would likely inactivate

⁹⁸ Skin inflammations characterized by rash and itchiness that develop on exposure to light. Usual presentation is cutaneous lesions on the face and dorsa (backs) of the hands. In severe cases, lesions necrotize and scar.

the enzyme, as would the drying step (see Section 3.2, Figure 2); moreover the process does not involve use of ethanol, acetone or methanol.

Ordinary commercial *Chlorella* products generally contain 2-3% dry weight Chl content (Jassby, 1988). *C. protothecoides* Krüger ACC⁹⁹ 25, however, has been shown to have no detectable Chl and diminished chloroplast structures¹⁰⁰ when “glucose-bleached” in high glucose/low nitrogen medium (Grant and Hommersand, 1974). WAP is generated from *C. protothecoides* S106 (UTEX 250), a strain that is identical to *C. protothecoides* UTEX 25 (S485) which is also known as *C. protothecoides* Krüger ACC NO. 25.¹⁰¹ Chl is absent and chloroplasts are minimized when this strain is grown in (and isolated from) a nitrogen-limited medium; as is the case in the manufacture of HLAF (Wu, 1994; Szabo *et al.*, 2012). Although Chl and, presumably, chloroplasts are present in the algal cells and subsequent WAP ingredient under the medium conditions of the WAP manufacturing process, the process includes several heating steps likely to inactivate the chlorophyllase enzyme and no process steps involve solvents associated with increased production of Pheides. Consistent with these safeguards, total PheideA concentration has been measured in WAP at 0.0334 mg/g (FDA, 2012; Szabo *et al.*, 2013), a concentration considerably lower than the limit of < 1.2 mg/g established by the Japanese Public Health Ministry in 1981(Becker, 1994; FDA, 2012).

7. EVALUATION

WAP is a whole algal protein composed of the dried biomass of the microalgae *Chlorella protothecoides* S106. WAP can be used as a protein source, analogous to soy- and animal-based proteins, in a variety of foods.²

WAP is stable for up to 18 months when stored at -20 °C or at ambient temperatures (23 ± 3 °C). WAP is stable for up to three months when stored at 40 °C.

In a published 28-day short-term repeated-dose study in rats, statistically significant effects were noted for several endpoints; these, however, were not found to be related to administration of the biomass material (a ground, yellow, high-lipid *C. protothecoides*). Under the conditions of the study, the NOAEL for the biomass material in the diet was 100,000 ppm, the highest dietary concentration provided, which corresponds to a dietary NOAEL of 7557 mg/kg bw/day in male rats and 8068 mg/kg bw/day in female rats. In corroborative support of these findings, an unpublished 28-day short-term repeated-dose study in hamsters demonstrated that consumption of *C. protothecoides* at up to ~6000 mg/kg bw/day was safe.

In a published 13-week subchronic toxicity study examining WAP in rats, statistically significant effects were identified for several endpoints; none, however, were attributable to ingestion of WAP because they were sporadic, not toxicologically or clinically relevant, and not

⁹⁹ Algal Culture Collection at Indiana University, Bloomington, IN.

¹⁰⁰ As measured by absorbance, Chl returns to normal levels within 48 hr of providing a “greening” medium (little/no glucose, high nitrogen) (Grant and Hommersand, 1974).

¹⁰¹ UTEX 25 was initially designated as ACC No. 25 when the collection was held by Indiana University. In 1976 the ACC at Indiana University was transferred to the University of Texas and became the core of the UTEX collection. <<http://www.sbs.utexas.edu/utex/insideUtex.aspx>>; site accessed December 12, 2011.

test article-related. Under the conditions of this study, the NOAEL for WAP in the diet was 100,000 ppm, the highest dietary concentration provided, which corresponds to a dietary NOAEL of 4805 mg/kg bw/day in male rats and 5518 mg/kg bw/day in female rats. Statistically significant effects were also noted for several endpoints in a published 13-week subchronic toxicity study examining the related HLAf in rats; these were sporadic, not toxicologically relevant, and not test article-related. Under the conditions of this study, the NOAEL for HLAf in the diet was 100,000 ppm, the highest dietary concentration provided, which corresponds to a dietary NOAEL of 4807 mg/kg bw/day in male rats and 5366 mg/kg bw/day in female rats.

In a study exploring the effect of consuming live cells or the isolated cell walls of *C. protothecoides* 902 (20-23 g/kg bw/day) on the $t_{1/2}$ of chlordecone in C^{14} -chlordecone-treated rats, no adverse effects associated with consumption of the living cells or the cell walls of the algae were reported by the authors.

No viable counts of *C. protothecoides* S106 were detected in any of the examined tissues from a three-week pathogenicity study in which rats were acutely dosed with live *C. protothecoides* S106 ($> 2.5 \times 10^8$ CFU/rat). Under the conditions of this unpublished, but corroborative, study, *C. protothecoides* S106 was not pathogenic.

The results of the bacterial reverse mutation and *in vivo* bone marrow chromosome aberration assays indicate that WAP is neither mutagenic nor clastogenic. The results of bacterial reverse mutation and *in vivo* bone marrow chromosome aberration assays also indicated that the related HLAf material was not mutagenic or clastogenic.

Under the conditions of the HRIP test in healthy male and female human subjects, WAP did not induce contact sensitization (allergic contact dermatitis) in any subject completing the study, although mild-to-moderate irritation was identified in several subjects. The results of the study therefore indicate a low likelihood of food allergy response to consumption of WAP. Under the conditions of a similar HRIP test in healthy male and female human subjects, the closely related HLAf material did not induce contact sensitization (allergic contact dermatitis) in any subject completing the study, although mild-to-moderate irritation was identified in several subjects. The results of this study also indicated low likelihood of food allergy response to consumption of HLAf.

Human infection with *C. protothecoides* is extremely unlikely. Only one case of human chlorellosis has been documented in the literature and this infection was localized and cutaneous, the result of surgical wounds exposed to contaminated water.

Assay of Solazyme's high-protein *C. protothecoides* biomass reported a PheideA concentration of 0.0334 mg/g (Day *et al.*, 2009), a concentration that is below the permitted limit of 0.8 mg/g for existing PheideA in algal preparations and well below the limit of < 1.2 mg/g for total potential PheideA imposed by the Japanese Public Health Ministry. The results of the conducted assay indicate that insufficient PheideA is present in WAP for photosensitivity to occur.

The NOAEL for WAP in a 13-week subchronic toxicity study in male and female rats was 100,000 ppm, the highest dietary concentration provided. The dietary NOAEL for WAP in

male and female rats was 4805 and 5518 mg/kg bw/day, respectively (equivalent to 288,300 mg/day in a 60-kg human).

The estimated daily intake of WAP, based on the 90th percentile WAP consumption levels from foods supplemented with WAP, has been determined to be 5.56 g/day. The potential theoretical maximum WAP consumption at the 90th percentile therefore may reach 5.56 g/day. This theoretical intake level represents a conservative estimate because it is unlikely that an individual would consume WAP from conventional foods at the 90th percentile level.

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8. CERTIFICATION

The undersigned authors of this document—a dossier in support of GRAS status determination for use of WAP—hereby certify that, to the best of their knowledge and belief, this document is a complete and balanced representation of all available information, favorable as well as unfavorable, known by the authors to be relevant to evaluation of the substance described herein.

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9. CONCLUSION

After critically evaluating the information available, as detailed within this dossier, the Expert Panel has determined that, based on common knowledge throughout the scientific community knowledgeable about the safety of substances directly or indirectly added to food, there is reasonable certainty that WAP, produced in accordance with current Good Manufacturing Practice (cGMP), and meeting the defined specifications, is safe under the intended conditions of use, and is also Generally Recognized As Safe (GRAS), by scientific procedures, when used as a protein source analogous to soy- and animal-based proteins in those foods without a standard of identity and in the food categories of baked goods and mixes, breakfast cereals, meal replacements, cheeses, milk products, dairy and nondairy products, egg products, fish products, meat products, poultry products, plant protein products, grain products and pastas, gravies and sauces, salad dressings, margarines, processed vegetables and vegetable juices, fresh and processed fruit juices, nonalcoholic beverages, gelatins and puddings, frozen dairy, soups, nut products, snack foods and soft candies, so that total daily consumption of WAP from all sources is up to 5.56 g WAP/day for 90th percentile consumers. In particular, the Expert Panel has evaluated the proposed use of WAP at specified levels in the foods listed in APPENDIX 2 of this document and has concluded that such use results in an estimated intake that is below 5.56 g/day WAP and is therefore Generally Recognized As Safe (GRAS).

It is our opinion that other experts qualified by scientific training and experience to evaluate the safety of food and food ingredients would concur with these conclusions.

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10. SIGNATURES

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11. REFERENCES

- Becker, E. W. (1994) Chemical composition. *In Microalgae: Biotechnology and Microbiology*. Cambridge University Press, Cambridge, England. p. 177-249.
- Becker, E. W. (2007) Micro-algae as a source of protein. *Biotechnology Advances* 25:207-210.
- Berger, R. S. and Bowman, J. P. (1982) A reappraisal of the 21-day cumulative irritation test in man. *Journal of Toxicology - Cutaneous and Ocular Toxicology* 1:109-115.
- Bernstein, I. L. and Safferman, R. S. (1966) Sensitivity of skin and bronchial mucosa to green algae. *Journal of Allergy* 38:166-173.
- Bernstein, I. L. and Safferman, R. (1973) Clinical sensitivity to green algae demonstrated by nasal challenge and *in vitro* tests of immediate hypersensitivity. *The Journal of Allergy and Clinical Immunology* 51:22-28.
- Brown, M. R. and Jeffery, S. W. (1992) Biochemical composition of microalgae from the green algal classes Chlorophyceae and Prasinophyceae. 1. amino acids sugars and pigments. *Journal of Experimental Marine Biology and Ecology* 161:91-113.
- Chandler, F. W., Kaplan, W. and Callaway, C. S. (1978) Differentiation between *Prototheca* and morphologically similar green algae in tissue. *Archives of Pathology and Laboratory Medicine* 102:353-356.
- Chrisostomou, A., Moustaka-Gouni, M., Sgardelis, S. and Lanaras, T. (2009) Air-dispersed phytoplankton in a mediterranean river-reservoir system (aliakmon-polyphytos, Greece). *Journal of Plankton Research* 31:877-884.
- Christaki, E., Florou-Paneri, P. and Bonos, E. (2011) Microalgae: a novel ingredient innutrition. *International Journal of Food Science and Nutrition* 62:794-799.
- Chung, B. Y., Kim, H. O., Park, C. W. and Lee, C. H. (2010) Diagnostic usefulness of the serum-specific IgE, the skin prick test and the atopy patch test compared with that of the oral food challenge test. *Annals of Dermatology* 22:404-411.
- Conte, M. V. and Pore, R. S. (1973) Taxonomic implications of Prototheca and Chlorella cell wall polysaccharide characterization. *Archives of Microbiology* 92:227-.
- Cudowska, B. and Kaczmarek, M. (2005) Atopy patch test in the diagnosis of food allergy in children with atopic eczema dermatitis syndrome. *Roczniki Akademii Medycznej w Białymstoku (1995)* 50:261-267.
- Dam, R., Lee, S., Fry, P. C. and Fox, H. (1965) Utilization of algae as a protein source for humans. *The Journal of Nutrition* 86:376-382.

- Day, A. G., Brinkmann, D., Franklin, S., Espina, K., Rudenko, G., Roberts, A. and Howse, K. S. (2009) Safety evaluation of a high-lipid algal biomass from *Chlorella protothecoides*. *Regulatory Toxicology and Pharmacology* 55:166-180.
- Draaisma, R. B., Wijffels, R. H., Slegers, P. M., Brentner, L. B., Roy, A. and Barbosa, M. J. (2013) Food commodities from microalgae. *Current Opinion in Biotechnology* 24:169-177.
- Durrant, N. W. and Jolly, R. D. (1969) Green algae, *chlorella*, as a contributor to the food supply of man. *Fish Ind. Research* 5:67-83.
- FAO (2013) Findings and recommendations of the 2011 FAO Expert Consultation on protein quality evaluation in human nutrition. In *FAO Food and Nutrition Paper 92. Dietary Protein Quality Evaluation in Human Nutrition: Report of an FAO Expert Consultation*(March 31 to April 2, 2011; Auckland, New Zealand). Food and Agriculture Organization of the United Nations (FAO), Rome. p. 19-50.
- FDA (2012) GRAS Notice Inventory: GRN No. 384. Algal oil derived from *Chlorella protothecoides* strain S106 (Cp algal oil) <<<http://www.accessdata.fda.gov/scripts/fcn/fcnNavigation.cfm?rpt=grasListing>>>, (site visited October 11, 2013).
- FDA (2013) GRAS Notice Inventory GRN No. 469 - *Chlorella protothecoides* strain S106 flour with 40-70% lipid (algal flour) <<<http://www.accessdata.fda.gov/scripts/fcn/fcnDetailNavigation.cfm?rpt=grasListing&id=469>>>, (site visited October 11, 2013).
- Fernández-Sevilla, J. M., Ación Fernández, F. G. and Molina Grima, E. (2010) Biotechnological production of lutein and its applications. *Applied Microbiology and Biotechnology* 86:27-40.
- Ferris, M. J., Sheehan, K. B., Köhl, M., Cooksey, K., Wigglesworth-Cooksey, B., Harvey, R. and Henson, J. M. (2005) Algal species and light microenvironment in a low-pH, geothermal microbial mat community. *Applied and Environmental Microbiology* 71:7164-7171.
- Genitsaris, S., Kormas, K. A. and Moustaka-Gouni, M. (2011) Airborne algae and cyanobacteria: Occurrence and related health effects. *Front. Biosci.* E3:772-787.
- Grant, N. G. and Hommersand, M. H. (1974) The respiratory chain of *Chlorella protothecoides*. *Plant Physiology* 54:50-56.
- Graziani, G., Schiavo, S., Nicolai, M. A., Buono, S., Fogliano, V., Pinto, G. and Pollio, A. (2013) Microalgae as human food: chemical and nutritional characteristics of the Thermoacidophilic microalga *Galdieria sulphuraria*. *Food & Function* 4:144-152.
- Harding, S. and Jones, P. (2008) Biological Evidence for the Insulin Independent Lowering of Blood Glucose by *Chlorella protothecoides*. Unpublished Report. p. 1-6.

- Hendler, S. S. and Rorvik, D. (2008) Chlorella. *In PDR for Nutritional Supplements*. 2nd Edition. Thomas Reuters, Montvale, NJ. p. 132-134.
- Hörtensteiner, S. (1999) Chlorophyll breakdown in higher plants and algae. *Cellular and Molecular Life Sciences* 56:330-347.
- Hörtensteiner, S., Chinner, J., Matile, P., Thomas, H. and Donnison, I. S. (2000) Chlorophyll breakdown in *Chlorella protothecoides*: characterization of degreening and cloning of degreening-related genes. *Plant Molecular Biology* 42:439-450.
- Huss, V. A., Ciniglia, C., Cennamo, P., Cozzolino, S., Pinto, G. and Pollio, A. (2002) Phylogenetic relationships and taxonomic position of Chlorella-like isolates from low pH environments (pH < 3.0). *BMC Evolutionary Biology* 2:13.
- Huss, V. A. R., Frank, C., Hartmann, E. C., Hirmer, M., Kloboucek, A., Seidel, B. M., Wenzeler, P. and Kessler, E. (1999) Biochemical taxonomy and molecular phylogeny of the genus *Chlorella sensu lato* (Chlorophyta). *Journal of Phycology* 35:587-598.
- ICH Expert Working Group (1996) ICH Harmonized Tripartite Guideline. Guideline for Good Clinical Practice E6(R1). International Conference of Harmonization (ICH). p. 1-53.
- Jagielski, T. and Lagneau, P. E. (2007) Protothecosis. A pseudofungal infection. *Journal de Mycologie Medicale* 17:261-270.
- Jassby, A. (1988) Some public health aspects of microalgal products. *In Algae and Human Affairs*. (C. A. Lembi and J. R. Waaland, Eds.) Cambridge University Press, p. 181-202.
- Jitsukawa, K., Suizu, R. and Hidano, A. (1984) *Chlorella* photosensitization. New phytophotodermatitis. *International Journal of Dermatology* 23:263-268.
- Kay, R. A. (1991) Microalgae as food and supplement. *Critical Reviews in Food Science and Nutrition* 30:555-573.
- Kessler, E. and Huss, A. R. (1992) Comparative physiology and biochemistry and taxonomic assignment of the *Chlorella* (Chlorophyceae) strains of the Culture Collection of the University of Texas at Austin. *Journal of Phycology* 28:550-553.
- Krcmery Jr., V. (2000) Systemic chlorellosis, an emerging infection in humans caused by algae. *International Journal of Antimicrobial Agents* 15:235-237.
- Lee, S. K., Fox, H. M., Kies, C. and Dam, R. (1967) The supplementary value of algae protein in humans diets. *Journal of Nutrition* 92:281-285.
- McNamee, P. M., Api, A. M., Basketter, D. A., Frank Gerberick, G., Gilpin, D. A., Hall, B. M., Jowsey, I. and Robinson, M. K. (2008) A review of critical factors in the conduct and

interpretation of the human repeat insult patch test. *Regulatory Toxicology and Pharmacology* 52:24-34.

Nasseri, A. T., Rasoul-Amini, S., Morowvat, M. H. and Ghasemi, Y. (2011) Single cell protein: production and process. *American Journal of Food Technology* 6:103-116.

Neil, L. L., McCullough, C. D., Lund, M. A., Evans, L. H. and Tsvetnenko, Y. (2009) Toxicity of acid mine pit lake water remediated with limestone and phosphorus. *Ecotoxicology and Environmental Safety* 72:2046-2057.

Peron, A., Tenconi, R., Leone, M., Macellaro, P., Ceriani, E. and D'Arcais, A. F. (2011) Negative atopy patch test and negative skin prick test reduce the need for oral food challenge in children with atopic dermatitis. *Pediatric, Allergy, Immunology, and Pulmonology* 24:107-112.

Pore, R. S. (1984) Detoxification of chlordecone poisoned rats with chlorella and chlorella derived sporopollenin. *Drug and Chemical Toxicology* 7:57-71.

Powell, R. C. and Nevels, E. M. (1961) Algae feeding in humans. *The Journal of Nutrition* 75:7-12.

Ramírez-Romero, R., Rodríguez-Tovar, L. E., Nevárez-Garza, A. M. and López, A. (2010) *Chlorella* infection in a sheep in Mexico and minireview of published reports from humans and domestic animals. *Mycopathologia* 169:461-466.

Ravishankar, G. A., Sarada, R., Kamath, B. S. and Namitha, K. K. (2006) Food applications of algae. In *Food Biotechnology*. (K. Shetty, G. Paliyath, A. Pometto and R. E. Levin, Eds.) 2nd Edition. CRC Press, Boca Raton, FL. p. 491-521.

Rawat, S., Agarwal, P. K., Choudhary, D. K. and Johri, B. N. (2005) Microbial diversity and community dynamics of mushroom compost ecosystem. In *Microbial Diversity: Current perspectives and potential applications*. (T. Satyanarayana and B. N. Johri, Eds.) I. K. International Publishing House, New Delhi, India.

Robinson, R. K. and Guzman-Juarez, M. (1978) The nutritional potential of the algae. *Plant Foods for Man* 2:195-202.

Satoh, K., Ooe, K., Nagayama, H. and Makimura, K. (2010) *Prototheca cutis* sp. nov., a newly discovered pathogen of protothecosis isolated from inflamed human skin. *International Journal of Systematic and Evolutionary Microbiology* 60:1236-1240.

Sirisansaneeyakul, S., Singhasuwan, S., Choorit, W., Phoopat, N., Garcia, J. L. and Chisti, Y. (2011) Photoautotrophic production of lipids by some chlorella strains. *Marine Biotechnology* 1-14.

- Skjånes, K., Rebours, C. and Lindblad, P. (2013) Potential for green microalgae to produce hydrogen, pharmaceuticals and other high value products in a combined process. *Critical Reviews in Biotechnology* 33:172-215.
- Solazyme Inc (2011)
Solazyme S106 strain comparison to *Auxenochlorella protothecoides* and *Chlorella vulgaris* strains, Unpublished report dated April 12, 2011.
- Solazyme Roquette Nutritionals LLC. (2012a) Whole Algal Cell Culture: An Acute Oral Toxicity Study In Rats. April 5, 2012. 1-139. Unpublished Work
- Solazyme Roquette Nutritionals LLC. (2012b) Validation of the viable count determination In rat matrices with *Chlorella Protothecoides*. January 11, 2012. Unpublished Report. 1-23. Unpublished Work
- Stackebrandt, E. and Goebel, B. M. (1994) Taxonomic note: A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *International Journal of Systematic Bacteriology* 44:846-849.
- Szabo, N., Matulka, R. A., Kiss, L. and Licari, P. (2012) Safety evaluation of a high lipid whole algalin flour (WAF) from *Chlorella protothecoides*. *Regulatory Toxicology and Pharmacology* 63:155-165.
- Szabo, N. J., Matulka, E. A. and Chan, T. (2013) Safety studies of whole algalin protein (WAP) from *Chlorella protothecoides*. *Food and Chemical Toxicology* 59:34-35.
- Tamura, Y., Maki, T. and Shimamura, Y. (1979) Causal substances of photosensitivity dermatitis due to chlorella ingestion (Hygienic studies on Chlorella. I). *Journal of the Food Hygienic Society of Japan* 20:173-180.
- Tap, R. M., Sabaratnam, P., Salleh, M. A., Razak, M. F. and Ahmad, N. (2012) Characterization of *Prototheca wickerhamii* isolated from disseminated algaemia of kidney transplant patient from Malaysia. *Mycopathologia* 173:173-178.
- Tartar, A., Boucias, D. G., Becnel, J. and Adams, B. J. (2003) Comparison of plastid 16S rRNA (rrn16) genes from *Helicosporidium* spp.: evidence supporting the reclassification of *Helicosporidia* as green algae (Chlorophyta). *International Journal of Systematic and Evolutionary Microbiology* 53:1719-1723.
- Tiberg, E. and Einarsson, R. (1989) Variability of allergenicity in eight strains of the green algal genus *Chlorella*. *International Archives of Allergy and Applied Immunology* 90:301-306.
- Tiberg, E., Rolfsen, W. and Einarsson, R. (1990a) Preparation of allergen extracts from the green alga *Chlorella*. Studies of growth variation, batch variation, and partial purification. *International Archives of Allergy and Applied Immunology* 92:23-29.

- Tiberg, E., Rolfsen, W., Einarsson, R. and Dreborg, S. (1990b) Detection of *Chlorella*-specific IgE in mould-sensitized children. *Allergy: European Journal of Allergy and Clinical Immunology* 45:481-486.
- Tiberg, E., Dreborg, S. and Björkstén, B. (1995) Allergy to green algae (*Chlorella*) among children. *Journal of Allergy and Clinical Immunology* 96:257-259.
- TNO (2012) TNO-Memorandum to Solazyme Roquette Nutritionals regarding TNO V9461 Final Report Assessment on the regulatory status of *Chlorella protothecoides* with regard to EU Regulation 258/97 EC on Novel Foods, dated January 2012. Report Number: V9461 p. 1-11.
- Tokusoglu, O. and Unal, M. K. (2003) Biomass nutrient profiles of three microalgae: *Spirulina platensis*, *Chlorella vulgaris*, and *Isochrysis galbana*. *Food Chemistry and Toxicology* 68:1144-1148.
- Torres, H. A., Bodey, G. P., Tarrand, J. J. and Kontoyiannis, D. P. (2003) Protothecosis in patients with cancer: case series and literature review. *Clinical Microbiology and Infection* 9:786-792.
- Trookman, N. S., Rizer, R. L. and Weber, T. (2011) Irritation and allergy patch test analysis of topical treatments commonly used in wound care: Evaluation on normal and compromised skin. *Journal of the American Academy of Dermatology* 64:S16-S22.
- Turjanmaa, K., Darsow, U., Niggemann, B., Rance, F., Vanto, T. and Werfel, T. (2006) EAACI/GA²LEN position paper: present status of the atopy patch test*. *Allergy* 61:1377-1384.
- Ueno, R., Hanagata, N., Urano, N. and Suzuki, M. (2005) Molecular phylogeny and phenotypic variation in the heterotrophic green algal genus *Prototheca* (Trebouxiophyceae, Chlorophyta). *Journal of Phycology* 41:1268-1280.
- UNPA (2011) New Old Dietary Ingredients List. United Natural Products Alliance (UNPA). p. 1-57.
- von Bergen, M., Eidner, A., Schmidt, F., Murugaiyan, J., Wirth, H., Binder, H., Maier, T. and Roesler, U. (2009) Identification of harmless and pathogenic algae of the genus *Prototheca* by MALDI-MS. *Proteomics Clinical Applications* 774-784:
- Wei, D., Chen, F., Chen, G., Zhang, X., Liu, L. and Zhang, H. (2008) Enhanced production of lutein in heterotrophic *Chlorella protothecoides* by oxidative stress. *Science in China, Series C: Life Sciences* 51:1088-1093.
- Wu, H. L., Hseu, R. S. and Lin, L. P. (2001) Identification of *Chlorella* spp. isolates using ribosomal DNA sequences. *Botanical Bulletin of Academia Sinica* 42:115-121.

- Wu, Q. Y. (1994) New discoveries in study on hydrocarbons from thermal degradation of heterotrophically yellowing algae. *Science in China, Series B* 37:326-335.
- Xiong, W., Li, X., Xiang, J. and Wu, Q. (2008) High-density fermentation of microalga *Chlorella protothecoides* in bioreactor for microbio-diesel production. *Applied Microbiology and Biotechnology* 78:29-36.
- Xiong, W., Liu, L., Wu, C., Yang, C. and Wu, Q. (2010) ¹³C-tracer and gas chromatography-mass spectrometry analyses reveal metabolic flux distribution in the oleaginous microalga *Chlorella protothecoides*. *Plant Physiology* 154:1001-1011.
- Yim, H. E., Yoo, K. H., Seo, W. H., Won, N. H., Hong, Y. S. and Lee, J. W. (2007) Acute tubulointerstitial nephritis following ingestion of *Chlorella* tablets. *Pediatric Nephrology* 22:887-888.

12. APPENDIX 1

Appendix 1. Materials used in the growth medium, fermentation process, recovery process, and antioxidants in the manufacture of WAP

Material	Use	Regulatory Status
Growth Medium Ingredients		
Dipotassium phosphate (K_2HPO_4)	Medium component	GRAS as a sequestrant under 21 CFR § 182.6285 when used in accordance with cGMP
Disodium phosphate (Na_2HPO_4)	Medium component	GRAS as a sequestrant under 21 CFR § 182.6290 when used in accordance with GMP
Potassium hydroxide (KOH)	Medium component	Affirmed as GRAS (21 CFR § 184.1631) as a pH control agent with no limitation other than cGMP
Sodium hydroxide (NaOH)	Medium component	Direct food substance affirmed as GRAS as a pH control agent under 21 CFR § 184.1763 when used in accordance with cGMP
Phosphoric acid (H_3PO_4)	Medium component	GRAS under 21 CFR § 182.1073 when used in accordance with cGMP
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	Medium component	Direct food substance affirmed as GRAS for use as a flavor enhancer, nutrient supplement, or processing aid with no limitation other than cGMP (21 CFR § 184.1443)
Ferric citrate ($Fe C_6H_5O_7$)	Medium component	Direct food additive affirmed as GRAS as a nutrient supplement with no limitation other than cGMP (21 CFR § 184.1298)
Industrol [®] 204	Medium component	Approved defoaming agent under 21 CFR § 173.340 when used in accordance with 21 CFR § 172.808
Hodag K-60-K	Medium component	Approved as an indirect food additive: Defoaming agent used in the manufacture of paper and paperboard (21 CFR § 176.210); Approved as an indirect food additive: Substances for use only as a component of adhesives (21 CFR § 175.105)
Cupric sulfate, pentahydrate ($CuSO_4 \cdot 5H_2O$)	Medium component	Direct food additive affirmed as GRAS as a nutrient supplement or processing aid with no limitation other than cGMP (21 CFR § 184.1261)
Ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$)	Medium component	Direct food substance affirmed as GRAS as a nutrient supplement or processing aid with no limitation other than cGMP (21 CFR § 184.1315)
Boric acid (H_3BO_3)	Medium component	Approved as an indirect food additive for paper and paperboard products in contact with dry food (21 CFR § 176.180); Prior sanctioned food ingredient used in the manufacture of paper and paperboard products used in food packaging (21 CFR § 181.30)
Zinc sulfate, heptahydrate ($ZnSO_4 \cdot 7H_2O$)	Medium component	GRAS as a nutrient under 21 CFR § 182.8997

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Appendix 1. Materials used in the growth medium, fermentation process, recovery process, and antioxidants in the manufacture of WAP

Material	Use	Regulatory Status
Manganese sulfate, monohydrate (MnSO ₄ ·H ₂ O)	Medium component	Affirmed as GRAS as a direct food substance with no limitation other than cGMP (21 CFR § 184.1461)
Sodium molybdate, dihydrate (Na ₂ MoO ₄ ·2H ₂ O)	Medium component	High purity (99.5 to 103.0%) and complies with the specifications of the American Chemical Society (ACS)
Nickel (II) chloride, 6-hydrate (NiCl ₂ ·6H ₂ O)	Medium component	Elemental nickel is affirmed as GRAS as a direct food substance (21 CFR § 184.1537) for use as a catalyst as defined in 21 CFR § 170.3(o)(24) with no other limitations than cGMP
Citric acid (C ₆ H ₈ O ₇) or Citric acid monohydrate (C ₆ H ₈ O ₇ ·H ₂ O)	Medium component	Direct food substance affirmed as GRAS with no limitations other than cGMP (21 CFR § 184.1033)
Calcium chloride (CaCl ₂) or Calcium chloride dihydrate (CaCl ₂ ·2H ₂ O)	Medium component	Direct food substance affirmed as GRAS as an anticaking agent, antimicrobial agent, curing or pickling agent, firming agent, flavor enhancer, humectant, nutrient supplement, pH control agent, stabilizer and thickener, surface-active agent, texturizer, and as a processing aid at levels not to exceed cGMP (21 CFR § 184.1193)
Thiamine hydrochloride (Vitamin B1)	Medium component	Direct food substance affirmed as GRAS with no limitations other than cGMP (21 CFR § 184.1875)
Biotin	Medium component	GRAS for use as a nutrient when used in accordance with cGMP (21 CFR § 182.8159)
Cyanocobalamin (Vitamin B12)	Medium component	Direct food additive that is affirmed as GRAS under 21 CFR § 184.1945
Calcium pantothenate, powder (D-Pantothenic acid hemicalcium salt)	Medium component	GRAS and permitted to be used in foods at levels not to exceed cGMP (21 CFR § 184.1212)
Aminobenzoic acid	Medium component	Ingredient approved for use (November 10, 1993) as an internal analgesic in drug products containing certain active ingredients offered over-the-counter (OTC) for certain uses CFR § 310.545(a)(23)(i)
Ammonium sulfate ((NH ₄) ₂ SO ₄)	Medium component	Direct food substance affirmed as GRAS for use as a dough strengthener, firming agent, and a processing aid when used at levels not to exceed cGMP (21 CFR § 184.1143)
Inositol (C ₆ H ₁₂ O ₆)	Medium component	Direct food substance affirmed as GRAS as a nutrient supplement and when used in special dietary foods with no limitations other than cGMP (21 CFR § 184.1370)
Choline chloride (C ₅ H ₁₄ ClNO)	Medium component	GRAS as a nutrient when used in accordance with cGMP (21 CFR § 182.8252)

Appendix 1. Materials used in the growth medium, fermentation process, recovery process, and antioxidants in the manufacture of WAP

Material	Use	Regulatory Status
Fermentation Process Ingredients		
Glycerol (glycerin) (C ₃ H ₈ O ₃)	Cryoprotectant to the master seed culture	Indirect food additive intended for repeated use in contact with food (21 CFR § 177.2420); Multiple purpose GRAS food substance when used in accordance with cGMP (21CFR § 182.1320)
Ammonium hydroxide (NH ₄ OH)	Control pH	Affirmed as GRAS (21 CFR § 184.1139) as a pH control agent with no limitation other than cGMP
Potassium hydroxide (KOH)	Control pH	Affirmed as GRAS (21 CFR § 184.1631) as a pH control agent with no limitation other than cGMP
Glucose as 95DE Corn syrup or equivalent	Prevent carbon starvation	Corn syrup, meeting the specification defined in 21 CFR § 168.120 (b), is a direct food substance affirmed as GRAS for use in food with no limitation other than cGMP
Glucose or Glucose monohydrate	Prevent carbon starvation	Corn sugar (aka, <i>D</i> -glucose, dextrose, [alpha]- <i>D</i> -glucopyranose) as the anhydrous or monohydrate: Direct food substance affirmed as GRAS (21 CFR § 184.1857) to be used in foods with no limitation other than cGMP
Antioxidants		
Ascorbic acid (C ₆ H ₈ O ₆)	Antioxidant; up to 2000 ppm	GRAS as chemical preservative (21 CFR § 182.3013)
Ascorbyl palmitate (C ₂₂ H ₃₈ O ₇)	Antioxidant; up to 2000 ppm	GRAS as chemical preservative (21 CFR § 182.3149)
Rosemary extract	Antioxidant; up to 200 ppm	Essential oils, oleoresins (solvent-free), and natural extractives (including distillates) affirmed as GRAS for intended use (21 CFR § 182.20)
Tertiary butylhydroquinone (TBHQ)	Antioxidant; up to 200 ppm	Food additive permitted for direct addition to food for human consumption as an antioxidant at levels such that the total antioxidant content of the food does not exceed 0.02% of the fat content of the food (21 CFR § 172.185)
Tocopherols, mixed	Antioxidant; up to 2000 ppm	GRAS as chemical preservatives (21 CFR § 182.3890)
Fortium® brand MTD10-WS Liquid Antioxidant (consists of canola oil, polysorbate 80, and natural mixed tocopherols)	Antioxidant	Canola oil: Direct food substance affirmed as GRAS (21 CFR § 184.1555); Polysorbate 80 (polyoxyethylene (20) sorbitan monooleate): Food additive permitted for direct addition to food for human consumption as an emulsifier when used in accordance with the specifications described in 21 CFR § 172.840; Tocopherols: GRAS as chemical preservatives (21 CFR § 182.3890)

Appendix 1. Materials used in the growth medium, fermentation process, recovery process, and antioxidants in the manufacture of WAP

Material	Use	Regulatory Status
Flow Agents		
Calcium silicate	Anti-caking agent	Food additive permitted for direct addition to food for human consumption when used as an anticaking agent in an amount not in excess of that reasonably required to produce its intended effect; not to exceed 2 % by weight of the food, except that it may be present up to 5 % by weight of baking powder (21 CFR § 172.410); GRAS as an anticaking agent when used at levels not exceeding 2 % in table salt and 5 % in baking powder in accordance with cGMP (21 CFR § 182.2227)
Silicon dioxide (amorphous silica)	Anti-caking agent	Food additive permitted for direct addition to food for human consumption when used as an anticaking agent used (1) in only those foods in which the additive has been demonstrated to have an anticaking effect, (2) in an amount not in excess of that reasonably required to produce its intended effect, (3) [Reserved], (4) in an amount not to exceed 2 % by weight of the food (21 CFR § 172.480)
Sodium aluminosilicate	Anti-caking agent	GRAS as an anti-caking agent for use at levels not exceeding 2 % in accordance with cGMP (21 CFR § 182.2727)
Amorphous silica – such as Zeofree 5162 (Huber Corp.); Aerosil 200 (Evonik Industries); Tixosil 331 (Rhodia Group)	Flow agents	Synthetic amorphous silica approved for use as an anticaking agent in food under 21 CFR § 172.480 at a maximum level of 2% by weight (GRN 00321); These three flow agents are also among those approved for use in EU at ≤1%

21 CFR = Title 21 of the Code of Federal Regulations; cGMP = current Good Manufacturing Process; EU =European Union; GRAS = Generally Recognized As Safe; GRN = GRAS notice number; WAP = Whole Algal Protein; ppm = parts *per* million

APPENDIX 2. Food items selected for WAP supplementation with intended levels of use

Food Product List	Parts per million (ppm)
MILK, SOY, READY-TO-DRINK, NOT BABY'S	10,000
MILK, IMITATION, FLUID, NON-SOY, SWEETENED, FLAVORS OTHER THAN CHOCOLATE	10,000
YOGURT, PLAIN, LOWFAT MILK	13,500
YOGURT, FROZEN, NS AS TO FLAVOR, LOWFAT MILK	13,500
CHOCOLATE SYRUP, REDUCED FAT MILK ADDED	13,500
FRUIT SMOOTHIE DRINK, MADE WITH FRUIT OR FRUIT JUICE AND DAIRY PRODUCTS	13,500
FLAVORED MILK DRINK, WHEY- AND MILK-BASED, FLAVORS OTHER THAN CHOCOLATE	10,000
INSTANT BREAKFAST, FLUID, CANNED	13,500
MEAL SUPPLEMENT OR REPLACEMENT, COMMERCIALY PREPARED, READY-TO-DRINK	13,500
INSTANT BREAKFAST, POWDER, NOT RECONSTITUTED	13,500
CREAM SUBSTITUTE, LIQUID	10,000
CREAM SUBSTITUTE, POWDERED	10,000
ICE CREAM, REGULAR, FLAVORS OTHER THAN CHOCOLATE	10,000
ICE CREAM, REGULAR, CHOCOLATE	10,000
LIGHT ICE CREAM, FLAVORS OTHER THAN CHOCOLATE (FORMERLY ICE MILK)	10,000
LIGHT ICE CREAM, CHOCOLATE (FORMERLY ICE MILK)	10,000
PUDDING, CHOCOLATE, READY-TO-EAT, NS AS TO FROM DRY MIX OR CANNED	10,000
PUDDING, FLAVORS OTHER THAN CHOCOLATE, READY-TO-EAT, NS AS TO FROM DRY MIX OR CANNED	10,000
IMITATION CHEESE, AMERICAN OR CHEDDAR TYPE	13,500
IMITATION MOZZARELLA CHEESE	13,500
SALMON CAKE OR PATTY	10,000
BEEF WITH VEGETABLE (DIET FROZEN MEAL)	10,000
CHICKEN AND NOODLES WITH VEGETABLE, DESSERT (FROZEN MEAL)	10,000
BEEF, BROTH, BOUILLON, OR CONSOMME	10,000
CHICKEN, BROTH, BOUILLON, OR CONSOMME	10,000
GELATIN DRINK, POWDER, FLAVORED, WITH LOW-CALORIE SWEETENER, RECONSTITUTED	10,000
GRAVY, POULTRY	10,000
GRAVY, BEEF OR MEAT	10,000
EGG SUBSTITUTE, NS AS TO POWDERED, FROZEN, OR LIQUID	32,000
SCRAMBLED EGG, MADE FROM POWDERED MIXTURE	13,500
BAKED BEANS, VEGETARIAN	10,000
HUMMUS	13,500
FALAFIL	10,000
PROTEIN POWDER, NFS	160,000
MEAL REPLACEMENT OR SUPPLEMENT, SOY- AND MILK-BASE, POWDER, RECONSTITUTED WITH WATER	16,000
HIGH PROTEIN BAR, CANDY-LIKE, SOY AND MILK BASE	16,000
TEXTURED VEGETABLE PROTEIN, DRY	160,000

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Food Product List	Parts per million (ppm)
MEAL REPLACEMENT OR SUPPLEMENT, LIQUID, SOY-BASED	160,000
TOFU, FROZEN DESSERT, FLAVORS OTHER THAN CHOCOLATE	13,500
BACON STRIP, MEATLESS	13,500
BREAKFAST LINK, PATTIE, OR SLICE, MEATLESS	64,000
CHICKEN, MEATLESS, NFS	64,000
FISH STICK, MEATLESS	13,500
FRANKFURTER OR HOT DOG, MEATLESS	13,500
VEGETARIAN BURGER OR PATTY, MEATLESS, NO BUN	13,500
SANDWICH SPREAD, MEAT SUBSTITUTE TYPE	10,000
VEGETARIAN CHILI (MADE WITH MEAT SUBSTITUTE)	13,500
TOFU AND VEGETABLES (INCLUDING CARROTS, BROCCOLI, AND/OR DARK-GREEN LEAFY VEGETABLES (NO POTATOES)), WITH SOY-BASED SAUCE (MIXTURE)	13,500
VEGETARIAN STEW	10,000
PEANUT BUTTER	10,000
BISCUIT MIX, DRY	13,500
BREAD, PITA	13,500
BRIOCHE	10,000
BAGEL	13,500
BREAD, MULTIGRAIN	13,500
BAGEL, MULTIGRAIN	13,500
MUFFIN, ENGLISH, MULTIGRAIN	13,500
MUFFIN, CHOCOLATE	13,500
CAKE BATTER, RAW, CHOCOLATE	13,500
CAKE BATTER, RAW, NOT CHOCOLATE	13,500
CHEESECAKE	13,500
CAKE, SPONGE, WITHOUT ICING	13,500
COOKIE, BROWNIE, WITHOUT ICING	13,500
PIE, CUSTARD	13,500
BREAKFAST BAR, NFS	13,500
MEAL REPLACEMENT BAR	80,000
SNACK BAR, OATMEAL	13,500
GRANOLA BAR, OATS, FRUIT AND NUTS, LOWFAT	13,500
GRANOLA BAR WITH NUTS, CHOCOLATE-COATED	13,500
CRACKER, SNACK	64,000
SALTY SNACKS, MULTIGRAIN, CHIPS	64,000
MULTIGRAIN MIXTURE, PRETZELS, CEREAL AND/OR CRACKERS, NUTS	10,000
PANCAKES, PLAIN	10,000
MACARONI, COOKED, NS AS TO FAT ADDED IN COOKING	10,000
OATMEAL, COOKED, REGULAR, FAT NOT ADDED IN COOKING	10,000
CORN FLAKES, NFS	13,500
CRISPY RICE	13,500
GRANOLA, NFS	13,500
100% BRAN	13,500

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Food Product List	Parts per million (ppm)
TOASTED OAT CEREAL	13,500
WHEAT, PUFFED, PLAIN	13,500
BEEF NOODLE SOUP	13,500
CHICKEN NOODLE SOUP	13,500
INSTANT SOUP, NOODLE	13,500
FRUIT JUICE BLEND, 100% JUICE	13,500
FRUIT SMOOTHIE DRINK, MADE WITH FRUIT OR FRUIT JUICE ONLY (NO DAIRY PRODUCTS)	13,500
VEGETABLE CHIPS	13,500
TOMATO AND VEGETABLE JUICE, MOSTLY TOMATO	10,000
SPAGHETTI SAUCE, MEATLESS	10,000
MUSHROOM SOUP, CREAM OF, PREPARED WITH MILK	64,000
VEGETABLE SOUP, PREPARED WITH WATER OR READY-TO-SERVE	64,000
BUTTER, WHIPPED, TUB, SALTED	10,000
MARGARINE, WHIPPED, TUB, SALTED	10,000
VEGETABLE OIL-BUTTER SPREAD, TUB, SALTED	10,000
CAESAR DRESSING	10,000
FRENCH DRESSING	10,000
MAYONNAISE-TYPE SALAD DRESSING	10,000
THOUSAND ISLAND DRESSING	10,000
GELATIN SNACKS	10,000
MILK CHOCOLATE CANDY, PLAIN	10,000
CHOCOLATE, SWEET OR DARK	10,000
CHOCOLATE, WHITE	10,000
RICE BEVERAGE	13,500
FRUIT FLAVORED DRINK, MADE FROM POWDERED MIX	13,500
FRUIT-FLAVORED THIRST QUENCHER BEVERAGE, LOW CALORIE	13,500
FLUID REPLACEMENT, ELECTROLYTE SOLUTION	13,500
ENERGY DRINK	32,000
FRUIT-FLAVORED BEVERAGE, DRY CONCENTRATE, WITH SUGAR, NOT RECONSTITUTED	13,500

NFS = Not further specified; NS = Not specified

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