NOMAD BIOSCIENCE GmbH
Biozentrum Halle
Weinbergweg 22
D-06120 Halle/Saale
Germany
Tel. 49 345 555 9887
Fax. 49 345 1314 2601

16 October, 2017

Antonia Mattia, Ph.D.
Director, Division of Biotechnology and GRAS Notice Review (HFS-255)
Office of Food Additive Safety
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740

Re: GRAS Notice for THAUMATIN Sweetener and Food Flavor Modifier

Dear Dr. Mattia,

Nomad Bioscience GmbH ("Nomad"; “Notifier”) is submitting this GRAS Notice for its **THAUMATIN sweetener and food flavor modifier** product. NOMAD has concluded, under FDA’s Final Rule pertaining to 21 CFR 170 (August 17, 2016), that the naturally occurring proteins comprising THAUMATIN are Generally Recognized as Safe (GRAS) for use as a sweetener and flavor modifier in various food products.

Thaumatin is comprised of several related proteins extracted from the Katemfe fruit of the native African bush *Thaumatococcus danielli*. The pulp of the arils of the fruit contain thaumatin proteins and has been used as a sweetener, flavor modifier and enhancer in native cuisines in West Africa for more than one hundred years. The active taste-modifying principles reside predominantly in the closely related proteins thaumatin I and thaumatin II. In the USA, EU, Japan and elsewhere, isolates of the Katemfe fruit have been marketed commercially since the mid-1990s under various trade names (e.g. Talin®; San Sweet T-100®), as low-calorie sweeteners and flavor modifiers. Thaumatin is listed in the Codex General Standard for Food Additives and permitted for general use in food (Codex Alimentarius 2016). In Europe, it is approved as a Sweetener or as a Flavour Enhancer under EFSA (E 957) for use in a number of different categories. In the USA, natural (extracted) thaumatin has GRAS classification (FEMA GRASa No. 3732; Oser 1984) as a flavor modifier, as does a recombinant version of the proteins ("thaumatin B-recombinant"; FEMA GRASa 3814; Smith 1996). In the EU and Japan, the acceptable daily intake limit (ADI) for thaumatin table top sweeteners is "not specified" because the proteins are considered to be non-toxic. Based on results of extensive preclinical safety studies and human clinical exposure, a working maximum level of 1.1 mg/kg-day, or 77 mg/person-day for a 70-kg person, has been adopted by the EU as a highly safe level for all subpopulations of consumers (EFSA 2015).

Thaumatin I and II proteins can be produced recombinantly in a wide range of hosts, including microorganisms such as bacteria and yeast, and in GM plants to enhance the organoleptic properties. Nomad is expressing thaumatin proteins in alternative host plants, but the product THAUMATIN is provided in purified form for direct application to foods and beverages as a sweetener and flavor modifier.

The amino acid sequences of Nomad's Thaumatin I and II are identical to the sequences of the same proteins extracted and purified from the Katemfe fruit. The level of purity of Nomad's thaumatins is at least as high as those of the proteins isolated from the arils of *Thaumatococcus*. Because they are nature-identical, Nomad's thaumatins have the same flavor-modifying profile as thaumatins derived from *Thaumatococcus*, and can be used at the same rate of application as the native thaumatins in various foods. Hence the intake of Nomad's thaumatins in human diets based on flavor functionality is expected to be equivalent to the intake from approved uses of current commercial products.

Using scientific procedures, Nomad has determined that its plant-produced THAUMATIN product is safe for use in various foods and beverages as a sweetener, flavor modifier and/or flavor enhancer, singly or in combination with other sweeteners or food flavor modifiers, at an intake up to the 1.1 mg/kg-day (77 mg/person-day for a 70-kg person) level considered safe by a high margin for all subpopulations.

In the current Notice, Nomad documents the identity, manufacturing process, product quality, safety, dietary exposure and potential risks from consumption of its THAUMATIN product, based on the company's own results and from publically available information.

Our submission complies with the 7-part format prescribed by FDA in its Final Rule for the GRAS Notice process (August 17, 2016), and includes a CD containing PDFs of the following documents:

1. FDA Form 3667 Nomad Bioscience GRN for THAUMATIN sweetener and food flavor modifier
2. GRN for THAUMATIN sweetener and food flavor modifier (Parts 1-7), which includes:
   APPENDIX A: Nomad THAUMATIN Safety Data Sheet
   APPENDIX B: THAUMATIN Manufacturing Process
   APPENDIX C: THAUMATIN Characterization
3. Copies of references used in the GRN

If the Agency has any questions or requires additional information to aid their review of Nomad’s findings and conclusions, please contact us at the address listed above. For convenience, you may also contact our regulatory and product development representatives in the USA, Dr. Kristi Smedley at Center for Regulatory Services Inc., Woodbridge, VA (Tel 703-590-7337; Email smedley@cfr-services.com), or Dr. Daniel Tusé at DT/Consulting Group, Sacramento, CA (Tel 707-290-9528; Email daniel@dt-cg.com).

Sincerely,

(b) (6)

Yuri Gleba, Ph.D.
Chief Executive Officer
Nomad Bioscience GmbH

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1. General Introduction and Claim of Exemption from Premarket Approval Requirements

Nomad Bioscience GmbH ("Nomad"; Notifier) THAUMATIN product is produced using a plant-based manufacturing process to match the amino acid sequence of naturally occurring thaumatin I and II proteins. Thaumatins (both extracted and recombinant) have been extensively studied scientifically for many decades, and a large public database of information exists regarding manufacturing methods, functionality, applications and safety. This extensive public record enables comparison of Nomad's new product to the currently available thaumatin-containing products. Results of a literature search and analysis of publicly available information on which Nomad based its conclusions of the safety of its THAUMATIN product are included in this Notice.

Native thaumatins found in the arils of the fruit of *Thaumatococcus danielli* have been used for centuries in West Africa as sweeteners and flavor modifiers. There is no ethnobotanical record of ill effects associated with consumption of impure thaumatins.

Industrially produced thaumatin-containing products have enjoyed an excellent safety record since their first use in human foods in the mid-1980s with expanded approvals in the 1990s. Nomad's plant-made thaumatins are identical in amino acid composition, sequence and structure to the native thaumatins. The purity of Nomad's THAUMATIN product is equal to or higher than that of the thaumatins prepared by extraction of *Thaumatococcus*. Thus, we project that THAUMATIN can be used safely at the same dietary intake levels as those of native thaumatins.

This Notice provides exposure estimates from consumption of foods that would be treated with Nomad's THAUMATIN, and includes a corresponding risk assessment. Notifier concludes that under the conditions of use described herein, THAUMATIN is generally recognized as safe and therefore should be exempt from premarket approval procedures under 21 CFR 170.36(a)(I). THAUMATIN is not intended for use in infant formulas.

1.1. Submission of Notice

This Notice is submitted in compliance with Subpart E of FDA's Final Rule of the GRAS Notification process (August 17, 2016) 21 CFR 170.203-170.285.

1.2. Name and Address of Notifier

NOMAD BIOSCIENCE GmbH
Biozentrum Halle
Weinbergweg 22
D-06120 Halle/Saale, Germany
Office: 49 345 555 9887
Fax: 49 345 1314 2601

Notifier's US Representative
Kristi O. Smedley, Ph.D.
Center for Regulatory Services, Inc.
5200 Wolf Run Shoals Rd.
Woodbridge, VA 22192
Office: 703-590-7337; Mobile: 703-786-7674
Fax: 703-580-8637
eMail: smedley@cfr-services.com

Notifier's US Representative
Daniel Tusé, Ph.D.
DT/Consulting Group
2695 13th Street
Sacramento, CA 95818
Telephone: 707-290-9528
Fax: 916-822-4124
Email: daniel@dt-cg.com

1.3. Common or Usual Name of the Notified Substance

THAUMATIN

1.4. Conditions of Use

Native thaumatin is comprised of several related proteins, but the major flavor-modifying properties reside in two very similar proteins, thaumatin I and thaumatin II. Thaumatins are naturally occurring proteins that can modify taste perception contextually, depending on their concentration and the properties of the food matrix into/onto which the thaumatins are applied. Nomad's THAUMATIN product is comprised of only thaumatin I and/or thaumatin II.

Notifier's product "THAUMATIN" is formulated to contain purified thaumatin I and/or thaumatin II produced individually in plant hosts, and can be blended to the same ratios as preparations of thaumatins from native sources. For comparison, a listing of the functionalities, use rates, and safety attributes of thaumatins when used as intended, including the source of supporting information, is provided in Table 7-1.

THAUMATIN is intended to modify the flavor of foods by imparting a sensation of sweetness, or by minimizing bitter, sour or other less desirable flavors, in various foods and beverages. As with the current thaumatin products, different levels of application are anticipated depending on the end use. Because Nomad's thaumatin proteins are compositionally identical and equal to or higher in purity as current sources of thaumatins, we anticipate their use at the same levels of application as the currently available thaumatins. As such, we estimate that the currently suggested intake of up to 1.1 mg/kg-day (body weight basis for all age groups, EFSA E 957 October 28, 2015) for existing thaumatins should also apply to Nomad's product.

Additional intake and safety information for subpopulations potentially consuming THAUMATIN, including children, adults and the elderly, is provided in Section 3 (Dietary Exposure) of this Notice.

1.5. Statutory Basis for Notifier's GRAS Conclusion

The statutory basis of the GRAS status in through scientific procedures in accordance with 21 CFR 170.30(b): GRAS Conclusion.

In accordance with the information provided in this Notice, it is Nomad Bioscience's conclusion that THAUMATIN is generally recognized as safe when used to modify the flavor profile of foods and beverages at application rates that would collectively amount to not more than 1.1 mg/kg-day (body weight basis) additive exposure in a typical human diet.

1.6. Not Subject to Preclearance

Notifier has concluded that THAUMATIN as manufactured via its plant-based process is generally recognized as safe, and as such the substance is not subject to pre-market approval requirements of the Federal Food Drug and Cosmetic Act.

1.7. Availability of Information for FDA Review

All data and information that serve as a basis for the GRAS conclusions are included in this Notice.

1.8. Public Disclosure

The information provided in this Notice is publically available and not subject to exception under 170.225(c)(8). All information contained in this Notice can be shared without restriction.

1.9. Certification

On behalf of Nomad Bioscience GmbH (Notifier), I certify that to the best of my knowledge, this GRAS Notice is complete, representative, and balanced with respect to the information provided, favorable or unfavorable, known to me and pertinent to the evaluation of the safety and GRAS status of our THAUMATIN sweetener and food flavor modifier product.

(b) (6)

Yuri Gleba, Ph.D.
Chief Executive Officer
NOMAD BIOSCIENCE GmbH
Biozentrum Halle
Weinbergweg 22
D-06120 Halle/Saale
Germany

2. Identity, Method of Manufacture, Specifications, Technical Effect

2.1. Identity, Structural and Functional Information

Identity

THAUMATIN consists of thaumatin I and/or thaumatin II proteins. Several plant genera produce sweetening and flavor-modifying proteins (Gibbs 1996; Kant 2005; Masuda 2006; Witty 1998). The flavoring substance thaumatin is found in the fruits of the tropical bush *Thaumatococcus danielli* (Benth), an African plant named in 1839 after physician and explorer W.F. Daniell, who referred to it as 'Katemfe', its Yoruba name (Inglett 1968; Suami 1997). Thaumatin consists of a mixture of at least 6 flavor-modifying proteins. Van der Wel & Loeve (1972) separated two proteins from homogenates of the arils, which they named thaumatins I and II, by removing low molecular weight materials. Commercial production of thaumatin began in the 1970s by the company Tate & Lyle in the UK under the brand name 'Talin', which had a sweetness of 1,600-2,700 times that of an 7-10% solution of sucrose (van der Wel 1972). A similar, proprietary mixture was sold in Japan under the brand name 'San Sweet T-100.' The chemistry and practical applications of thaumatins have been extensively described in several original and review articles (e.g. Lee 1987; Kim 1988; Hough 1993; Shallenberger 1993; Witty 1998; Zemanek 1995; Suami 1997; Greenly 2003). Table 2-1 lists the sweetness ranking of thaumatins relative to sucrose (from Greenly 2003).

Thaumatins I and II have similar properties, amino acid composition, sweetness, molecular weight (both are ~22 kDa) and highly similar amino acid sequences, differing by only 5 amino acid residues. Each protein is a single polypeptide chain of 207 amino acids with 8 intramolecular disulphide linkages (Suami 1997). X-ray crystallography of thaumatin I revealed the features of the protein's backbone (de Vos 1985; Kim 1988; Ogata 1992). Circular dichroism studies (van der Wel 1984) showed few α-helices, but many β-pleated sheet strands and bends. It is thought that the constrained structure of thaumatins is responsible for inducing taste sensation. Either heat denaturation of thaumatins or cleavage of the disulphide bridges results in loss of sweetness, suggesting that the tertiary structure of the protein triggers taste modulation through a highly stereoselective process (Kaneko 2001; Hough 1993). A molecular diagram of thaumatin I is shown in Figure 2-1 (from Nagarajan 2017).

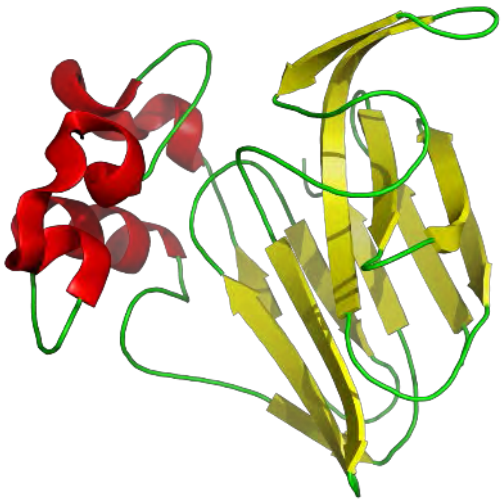

Figure 2-1. Structure of thaumatin I

Table 2-1. Relative sweetness of natural and artificial sweeteners compared to sucrose

Sweetener	Sweetness/sucrose
Neotame	13,000
Thaumatin	3,000
Alitame	2,000
DHC	2,000
Sucralose	600
Saccharin	300
Stevia	300
Acesulfame-K	200
Aspartame	180
Glycyrrhizin	100
Cyclamate	30
HFCS	1.5
Honey	1.5
Sucrose	1.0
Tagatose	1.0
Xylitol	1.0
HSH	0.9
Maltitol	0.9
Erythritol	0.7
Isomalt	0.65
Mannitol	0.5
Trehalose	0.5
Sorbitol	0.6
Lactitol	0.4

Structural Information for Plant-Made Thaumatin I and Thaumatin II

The comparative amino acid sequences of thaumatins I and II, including Notifier's plant-expressed thaumatins, are shown in Figure 2-2. Regardless of how they are obtained, the two proteins differ from each other by only 5 amino acids. Amino acid differences in specific positions are indicated in the figure in bold-face colored font (blue are amino acids found in thaumatin I; red are amino acids found in thaumatin II; adapted from Faus 2008). Notifier's plant-expressed, mature thaumatins I and II have identical amino acid sequences to the corresponding proteins from *Thaumatococcus*.

The sequences shown are for the mature proteins; thaumatins are natively expressed as pre-proproteins containing N-terminal signal and C-terminal peptide additions. These precursors are processed *in planta* to yield the intermediate proprotein devoid of the N-terminal peptide but still containing the C-terminal addition. Subsequent processing *in planta* yields the nature-identical sequence of amino acids in the mature, isolated thaumatin. Thaumatins do not contain glycosylation sites on their 207-aa backbone; hence, like the proteins derived from *Thaumatococcus*, Notifier's thaumatins are non-glycosylated polypeptides.

```
Thaumatin I     1  ATFEIVNRCSYTVWAAASKGDAALDAGGRQLNSGESWTINVEPGTNGGKIWARTDCYFDD
Thaumatin II    1  ATFEIVNRCSYTVWAAASKGDAALDAGGRQLNSGESWTINVEPGTKGGKIWARTDCYFDD

Thaumatin I    61  SGSGICKTGDCGGLLRCKRFGRPPTTLAEFSLNQYGKDYIDISNIKGFNVPMNFSPTTRG
Thaumatin II   61  SGRGICRTGDCGGLLQCKRFGRPPTTLAEFSLNQYGKDYIDISNIKGFNVPMDFSPTTRG

Thaumatin I   121  CRGVRCAADIVGQCPAKLKAPGGGCNDACTVFQTSEYCCTTGKCGPTEYSRFFKRLCPDA
Thaumatin II  121  CRGVRCAADIVGQCPAKLKAPGGGCNDACTVFQTSEYCCTTGKCGPTEYSRFFKRLCPDA

Thaumatin I   181  FSYVLDKPTTVTCPGSSNYRVTFCPTA
Thaumatin II  181  FSYVLDKPTTVTCPGSSNYRVTFCPTA
```

Figure 2-2. Amino acid sequences of thaumatin I and thaumatin II

A Safety Data Sheet (SDS) for THAUMATIN is found in APPENDIX A. The method applied to manufacture Notifier's thaumatin proteins is described in APPENDIX B. Analytical methods used to define the physicochemical properties of Notifier's thaumatins are summarized in APPENDIX C. Notifier's plant-made thaumatin I and II conform to their predicted compositions and share the amino acid sequences of the native *Thaumatococcus* proteins.

Quantitative Composition

THAUMATIN is prepared in bulk as a dry powder as described in **APPENDIX B.** The **Specification** for the bulk product is found in APPENDIX B, specifically Table B-1, as well as in Table 2-2**.** Typically, THAUMATIN is supplied as a solid of >98% purity. Thaumatins are highly water soluble (>20% w/v). The bulk product is dissolved/diluted in water or other suitable food-compatible vehicle or directly mixed into foods or beverages to achieve the desired effect. THAUMATIN can be used singly or in combination with other sweeteners, flavor modifiers, or other foods and food additives to modify or enhance the flavor profile of foods and beverages. The amount of THAUMATIN used is food- and application-specific and depends on the desired result that one is trying to achieve.

Mode of Action

Thaumatins interact with the sweet taste receptors in the tongue. Unlike sweeteners that have a single function, thaumatins also interact with other gustatory receptors. Thaumatins' effect on multiple taste receptors is contextual and depends on the presence of other flavors. Such a pluripotent effect explains the wide range of industrial applicability of thaumatins in flavor modification.

In addition to their history of human use by African and more recently by Western and Eastern cultures, thaumatins have been studied systematically for their flavor modifying properties at the molecular level as well as in animal studies *in vivo* and *ex vivo*. As defined in whole animal studies using various species and in isolated gustatory cells *in vitro*, thaumatins have a demonstrable effect on various groups of taste receptors and these interactions affect perception of taste (Bartoszewski 2003; Faus 2008; Glaser 2000; Greenly 2003; Hellekant 1996; Kaneko 2001; Kinghorn 1986; Lim 2012; Nagarajan 2017; Ohta 2011; Sardesai 1991; Schiffman 1995; Suami 1997; Tinti 2000; van der Wel 1972; Witty 1990). Thaumatins find multiple uses in food technology because these proteins can impart a sweet flavor, enhance desirable flavor notes, or mask or ameliorate unpleasant flavors in food components.

At the molecular level, the electrical charge distribution on the thaumatin molecules is believed to mediate the interaction of thaumatins with the taste receptors. This is demonstrated by the masking effects on the typically bitter taste of cations such as Na, Fe and K, and its simultaneous enhancement of other free ions, such as Cl^-. Thaumatins' effect in the presence of more complex flavor compounds suggests that flavor modification depends on the nature of the individual molecules present (Natex 2017). This is evident in thaumatins' effect with pronounced flavors such as peppermint, ginger, cinnamon and coffee, which it enhances while simultaneously reducing the fiery, peppery or bitter elements associated with them (Natex 2017). The strength of the interaction between the thaumatin molecules and the taste receptors may account for thaumatins' intensity and the duration of their flavor-modifying effects.

Natural thaumatin was found to consist of six closely related proteins (I, II, III, a, b and c), all with a molecular mass of ~22 kDa (207 amino acids) (van der Wel 1972; Ledeboer 1984). The three-dimensional structure of thaumatin I had been determined at high resolution (de Vos 1985; Ogata 1992), revealing that the protein consists of three domains: (a) an 11 strand, flattened β-sandwich (aa 1–53, 85–127 and 178–207, domain I); (b) a small disulfide-rich region (54–84, domain III); and (c) a large disulfide-rich region (128–177, domain II).

Modification of lysine residues in the structure of thaumatins was found to reduce their sweetness (Kaneko 2001). Phosphopyridoxylation of lysine residues Lys78, Lys97, Lys106, Lys137 and Lys187 markedly reduced sweetness. The intensity of sweetness was returned to that of native thaumatins by dephosphorylation of these phosphopyridoxylated lysine residues except Lys106. These lysine residues occurred in thaumatins, but not in non-sweet thaumatin-like proteins, suggesting that these lysine residues were required for sweetness. Masuda (2016) found that a D21N mutation enhanced thaumatin sweetness. Thaumatin I and II can lose their sweetness on heating at alkaline pH, but the proteins are stable at neutral to acidic pH (Lim 2012).

2.2. Method of Manufacture

The THAUMATIN manufacturing process is described in APPENDIX B. Notifier uses a **plant-based manufacturing process** for producing THAUMATIN proteins; the method is an adaptation of the process used to manufacture biopharmaceuticals, which have been administered in multiple clinical trials under FDA IND. The thaumatins are produced using recombinant technology to yield concentrated extracts. Host plants that are currently used in the manufacture of THAUMATIN include the food species **spinach** (*Spinacia oleracea*), **red beet** (*Beta vulgaris*) or **lettuce** (*Lactuca sativa*). These same hosts were found acceptable for the

manufacture of antimicrobial proteins as food safety processing aids, as described in Notifier's GRN 593 and GRN 676. The safety features of these host species were detailed in GRN 593.

The plant-derived biomass remaining after thaumatin protein extraction is treated and discarded (disposed) and is not used as a human food or animal feed product, additive or supplement.

2.3. Composition and Specification

Characteristic Properties

The characteristic properties of plant-made THAUMATIN are summarized in the Specification shown in Table 2-2. Because thaumatin I and II have equivalent sweetening and taste modifying properties, the THAUMATIN product may consist of either individual thaumatin I or thaumatin II protein, or a blend of thaumatin I and thaumatin II proteins mixed to the same final total thaumatin content.

Importantly, Notifier studied at the molecular level the allergenic potential of plant-expressed thaumatin I and II for use in food and determined, from published information, that Notifier's thaumatins should pose no higher allergenic risk than the *Thaumatococcus*-derived commercial thaumatins currently on the market (see Section 6.2 Allergenic Potential of Thaumatin for detail).

Formulation

THAUMATIN is provided in bulk as a dry powder, which the end-user can dissolve and/or dilute in water or other food-acceptable vehicle according to instructions and mixed in foods and beverages at levels sufficient to achieve the desired flavor modifications. On a molar basis, the taste-modifying effects of thaumatin I and II are interchangeable; THAUMATIN bulk can consist of either protein or a mixture thereof. The application rate for THAUMATIN is projected to be approximately the same as that of extracted thaumatins.

Content of Potential Human Toxicants in THAUMATIN

None. Thaumatin proteins comprise an approved product. Process impurities are low and formulation excipients are approved for food applications.

Specification

The Specification for THAUMATIN is shown in Table 2-2. The process used to manufacture the product with this specification is shown in APPENDIX B: THAUMATIN Manufacturing Process.

Table 2-2. Specification of THAUMATIN Product

THAUMATIN Bulk Product		
Parameter	**Specification limit**	**Method**
Appearance	Powder, yellowish to light tan	Visual
Minimum total thaumatin content (as percent of total protein)	≥98%	HPLC
Solubility (DI water)	600 mg/mL	Visual
pH of a 1% solution (excipient-dependent)	2.7 – 6.0	Potentiometric
Heavy metals (sum of Ag, As, Bi, Cd, Cu, Hg, Mo, Pb, Sb, Sn)	≤30 ppm	USP38<233>

Lead	≤5 ppm	USP38<233>
Bioburden	≤5,000 CFU total per g	USP32<61>
Agrobacterium per 10 g sample	0 (absent)	Selective plate-based assay
Undesirable microorganisms, including *Escherichia coli, Pseudomonas aeruginosa, Salmonella spp.* or coagulase-positive *Staphylococcus spp.*, per 25 g	0 (absent)	USP32<1111>
Stability (dry concentrate; 0-10°C)	>6 months	HPLC; thaumatin peak in 1% solution at T_n relative to T_0

2.4. Technical Effect and Suitability of Use

Notifier has determined that the intended use of THAUMATIN does not raise safety concerns; as such, physical or technical data are not required.

2.5. Overall Conclusion

The results of studies supporting this Notice show that thaumatins I and II can be produced consistently using recombinant expression in a variety of host plants. The recombinant thaumatins can be readily purified from plant biomass and exhibit the characteristic properties of extracted thaumatins.

The molecular composition of Notifier's thaumatins is the same as those of their native counterparts. The gene sequences used in the expression vectors encode protein precursors that are correctly processed *in planta* to yield the same mature thaumatin proteins that are isolated from *Thaumatococcus*, including identical amino acid sequences. Regardless of source, the mature thaumatin proteins lack glycosylation because the polypeptide backbones do not contain glycan addition sites.

The safety, physicochemical and taste-modifying properties of fermentation-derived recombinant thaumatins match those of native thaumatins (Smith 1996) and both are available commercially. Ample evidence of safety of thaumatins exists in the published literature. Thaumatin's approval for use in foods and beverages in EU countries, Japan, Australia, Israel and elsewhere underscore thaumatin's excellent record of safety obtained during more than 30-years of pre- and post-market surveillance.

In sum, the main difference between Notifier's plant-produced thaumatins and commercially available *Thaumatococcus*-extracted or fermentation-derived thaumatins is their method of manufacture.

3. Dietary Exposure

Thaumatin is comprised of several related proteins extracted from the Katemfe fruit of the native African bush *Thaumatococcus danielli*. The pulp of the arils of the fruit contain thaumatin proteins and has been used as a sweetener, flavor modifier and enhancer in native cuisines in West Africa for more than one hundred years. The active taste-modifying principles reside predominantly in the closely related proteins thaumatin I and thaumatin II. In the West, Japan, Australia, Israel and other countries, isolates of the Katemfe fruit have been marketed commercially since the mid-1990s under various trade names (e.g. Talin®; San Sweet T-100®), as low-calorie sweeteners and flavor modifiers. Thaumatin is listed in the Codex General Standard for Food Additives and permitted for general use in food (Codex Alimentarius 2016). In Europe, it is approved as a Sweetener or as a Flavour Enhancer under EFSA (E 957) for use in a number of different categories.

In the USA, natural (extracted) thaumatin has GRAS classification (FEMA GRASa No. 3732; Oser 1984) as a flavor modifier, as does a recombinant version of the proteins ("thaumatin B-recombinant"; FEMA GRASa

3814; Smith 1996). In the EU and Japan, the acceptable daily intake limit (ADI) for thaumatin table top sweeteners is "not specified" (*Quantum satis*) because the proteins are considered to be non-toxic.

Based on their flavor-modifying profile, thaumatins are used in the 1-400 ppm range, or 1-400 mg thaumatin/kg or /L food or beverage. When used at these levels and on the same foods, the use rates and dietary exposure of Notifier's product are expected to be the same as those for currently available thaumatin products. In the USA, FEMA's suggested application rates range from 1-150 ppm, because they exclude uses of thaumatin as a sweetener (Cohen 2016). Notifier used the more inclusive EU database for estimating the dietary intake of thaumatins from THAUMATIN, rather than domestic figures, because it represents a higher potential intake scenario (1-400 ppm) by covering all food categories and use rates encompassed by EU statutes (EFSA 2015). The upper level of exposure would be **77 mg/person-day**.

3.1. Estimated dietary intake of THAUMATIN in foods and beverages

With respect to human exposure, we expect the same levels of consumption of Notifier's thaumatins per weight or volume of treated food as those allowed for extracted thaumatins. For example, the European Food Safety Authority (EFSA) provides guidance on maximum permitted levels (MPL) of thaumatin on various foods and beverages according to Annex II to Regulation (EC) No 1333/2008 (OJEU 2011).

Thaumatin's EU food additive designation number is E 957. The MPL for E 957 ranges from a low of 5 mg/kg in "flavored fermented milk products" (e.g. yogurt) to a high of 400 mg/kg in "food supplements supplied in a syrup-type or chewable form" (EFSA 2015). The same regulations allow the unlimited addition of thaumatin (*Quantum satis*[1]) when the protein is used as a table top sweetener. It is envisioned that THAUMATIN could be used at equivalent application rates on various foods. Thaumatin is not considered a sweetener *per se* in the USA, but as a flavor enhancer or modifier FEMA cites use ranges from a low of 1 ppm for baked goods to a high of 150 ppm for chewing gum (Cohen 2016).

The application rates of thaumatin to various food products (maximum permissible level, MPL) were derived by assessing the consumption of each food type and estimating the additive intake to reach a maximum exposure of **1.1 mg thaumatin/kg body weight (bw) per day, or 77 mg/day for a 70-kg person.** Consumption values were derived from typical European diets, taking into consideration country specific dietary preferences in Belgium, Bulgaria, Czech Republic, Denmark, Finland, France, Germany, Greece, Italy, Latvia, Netherlands, Spain, Sweden and the UK and various population age groups (**adult, infant, elderly**).

The original application levels allowed by EFSA for a limited number of foods were first published in 1988, and later amended (expanded) in 2011 (EFSA 2011) to include more food types and higher allowed levels of thaumatin (see OJEU 2011 for detail). Revision of statutes for expanded use of thaumatin was based on thaumatin's functionality and post-market safety record. Additional detail is provided in Section 6.3.

3.2. Additional exposure to thaumatins (intake not related to THAUMATIN product)

With the goal of estimating a "highest intake" scenario for THAUMATIN, we assumed that Notifier's product (a) replaces all the current commercial *Thaumatococcus*-extracted and recombinant thaumatins, plus (b) expands the market to twice its current size via the availability of a new product with a competitive price and higher availability. A doubling of the current market is plausible, and is not an underestimate, because even though thaumatins have been commercially available for nearly 3 decades, their sales volume has been lower

[1] With respect to food additives, the term "*Quantum satis*" indicates that no maximum level is specified. However, additives must be used in accordance with good manufacturing practice, at a level not higher than is necessary to achieve the intended purpose and provided that they do not mislead the consumer.

than those of other sweeteners and flavor enhancers. To reduce speculation, it was also assumed that a higher volume of the same types of food groups would be treated at current application rates with thaumatins (i.e. no new food categories). With these assumptions, and using the more inclusive EU database, total consumer exposure per capita from Notifier's product plus existing sources of thaumatins would be 2 x 77 mg/day or approximately **160 mg/day**.

Perspective on the safety risks of such intake from Notifier's THAUMATIN product and from all sources of thaumatin is provided in Section 6.3 (Safety in Relation to Dietary Intake of THAUMATIN).

4. Information on Any Self-Limiting Levels of Use

THAUMATIN is approximately 3,000-times sweeter than sucrose (Table 2-1); therefore, a self-limiting level of use might be expected on the bases of (a) sensory saturation and (b) organoleptic tolerance.

5. Experience Based on Common Use in Food Before 1958

"Common use" depends on geography and cultural practices. In industrialized regions (e.g. USA, Europe, Japan, Australia, Israel) thaumatin preparations have been used in human and animal foods beginning in the late 1980s to mid-1990s, depending on the regulatory approval pathway in these countries. However, it is noted that naturally occurring thaumatins from *Thaumatococcus* have been used in indigenous human diets in West Africa for hundreds of years, as documented in British surgeon W.F. Daniell's report when he discovered the native use of *Thaumatococcus* pods in Nigeria in 1839 (Kinghorn 1986). Notifier's plant-produced THAUMATIN described in this Notice has not yet been commercialized.

6. Basis for Conclusion of THAUMATIN's GRAS Status

Notifier has used scientific procedures to conclude that its THAUMATIN sweetener and food flavor modifier product is GRAS under the conditions of intended use. Notifier has relied on detailed physicochemical analyses of its thaumatin proteins and on extensive comparisons of those properties to a large volume of public information on thaumatins, including thaumatin's excellent safety record since these proteins were first commercially used as sweeteners and food flavor modifiers in multiple countries.

Section 6 of this Notice summarizes the data and information on thaumatins that were used by Notifier to support its GRAS claims. In Section 7 (Supporting Data and Information), Table 7-1 presents a tabulated summary of this information from all sources.

6.1. THAUMATIN Overall Safety

Prior to their broad approval for consumer use in the EU, Japan and other nations, thaumatin proteins were subjected to extensive safety assessments in animals and in humans, which have led to definition of application rates and daily exposure limits. Guidelines for thaumatin use and dietary exposure limits have been published in a number of regulatory documents issued by the European Food Safety Authority (EFSA 2015), based on reviews conducted by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (JECFA 1983, 1986) and the EU Scientific Committee on Food (SCF 1985, 1989).

Thaumatin is considered safe for use in food and beverages. It is approved by EFSA in the EU (E 957) and in Japan, Australia, Israel and other countries as a food additive. In the USA thaumatin was granted GRAS status by FEMA (GRASa No. 3732, Oser 1984; and GRASa No. 3814, Smith 1996) as a flavor modifier, but not as a sweetener.

A discussion of thaumatin's safety from which a projection of Notifier's THAUMATIN safety can be drawn is presented herein.

Specifically, the safety profile of THAUMATIN's **active ingredients** can be deduced from (1) their equivalence to commercially available thaumatins, and (2) published results of detailed studies on the safety of thaumatin conducted over the last 30 years. The safety of the **gene expression system** and of residual **impurities from the host plants** that Notifier uses to produce THAUMATIN was discussed in detail in GRN 593 (GRN 593 Section A.2.2; pp 25-28); the processes described herein and in GRN 593 are analogous and differ mainly in the higher downstream purification of THAUMATIN.

With respect to thaumatin proteins, studies by Higginbotham et al. (1983) and Hagiwara et al. (2005) have been pivotal to establishing safety and have helped craft regulations for the application rate and safe usage levels of these substances. Higginbotham et al. (1983) found that thaumatin, when used as a sweetener/flavor enhancer, was unlikely to be hazardous at the anticipated level of consumption. They studied the safety of thaumatin over a 7-year period when various purities and formulations of thaumatin were under development and the initial product was being commercially manufactured. A summary of their findings includes:

Thaumatin digestibility. Thaumatin was readily digested prior to absorption in rats. Groups of 20 animals were split into 2 x 10 animals each, and fed diets containing no (0%) or 5.73 wt% thaumatin, for 10 consecutive days with sampling at two time periods. The results of metabolic analyses showed that thaumatin was 89 to >90% digested when fed as a mixed additive to the diet. Digestibility was high even though thaumatins I and II have 8 intramolecular disulfide bridges.

Subacute toxicity in rats and dogs. Thaumatin-containing diets were fed to groups of 20 male and 20 female rats, and to groups of 4 male and 4 female dogs, at concentrations of 0%, 0.3%, 1.0% and 3.0% (w/w), daily for 13 weeks. Behavioral, food consumption, weight gain, clinical chemistry, cytology, ophthalmoscopy, urinalysis, and histopathology observations were conducted on all animals at every dose group. No adverse effects were found for any dose of thaumatin administered.

Teratogenicity. Thaumatin was not teratogenic when administered orally by gavage to groups of 20 pregnant rats at 0, 200, 600 and 2,000 mg/kg body weight/day from day 6 to 15 of gestation. No thaumatin- or dose-related adverse effects on the fetus were found.

Mutagenicity. Thaumatin was evaluated for mutagenic potential using two standard assays, the dominant-lethal test, and the Ames' *in vitro* mutagenicity assay. In the first study, groups of 15 male mice each of proven fertility were intubated daily for 5 consecutive days with thaumatin or negative or positive controls. Thaumatin administration had no effect on the incidence of dominant lethal mutations when administered at doses of 200 and 2,000 mg/kg-day. In the second study, the lack of mutagenic potential was confirmed in bacterial mutagenicity assays with *Salmonella typhimurium* (strains TA1535, TA1537, TA1538, TA98 and TA100) and *Escherichia coli* WP2, at levels of addition of 0.05–50 mg thaumatin/plate. All results were negative for mutagenicity.

No observed adverse effect level. The multi-species preclinical studies by Higginbotham et al. helped establish the **NOAEL** (no observed adverse effect level) for thaumatins at **1.4** (dog) **to 3.0 g/kg** (rat). By conventional criteria, thaumatins are non-toxic. Allergenic potential was low in the risk scale (Section 6.2).

Persistence of thaumatin proteins and genes. To complement Higginbotham (1983), Szwacka (2012) studied digestibility of thaumatins and the uptake of the thaumatin genes in animals fed transgenic fruit. The proteins were fully digested, and there was no gene transfer to the intestinal microflora.

6.2. Allergenic potential of THAUMATIN

Because Notifier's thaumatin I and II are identical in amino acid sequence and no less pure than commercial compositions of thaumatins, the allergenic risk for the recombinant proteins is deemed to be not higher than that of approved commercial products. Internal and external data were used to reach this conclusion. Baniulis et al. (Baniulis 2008) had conducted a computational analysis of thaumatin II allergenicity and assessed potentially antigenic elements in thaumatin-like family proteins. Of the various candidate antigenic domains in thaumatin II, only four amino acid residues (Thr12, Leu74, Gln133 and Thr161) were found to possess high surface exposure and antigenic propensity, in theory. Using a similar technique, Notifier conducted an updated search *in silico* for potentially antigenic domains in both thaumatins I and II. The results are summarized below.

Allergenic domain searches *in silico*

The following amino acid sequences were analyzed by Notifier using the AllergenOnline database (Jan 18, 2017 version; accessed Aug 14, 2017; http://allergenonline.org/databasefasta.shtml) to assess whether Notifier's thaumatins shared aa sequences with known allergens, including newly identified allergens.

Thaumatin I (207 aa)

```
ATFEIVNRCSYTVWAAASKGDAALDAGGRQLNSGESWTINVEPGTNGGKIWARTDCYFDD
SGSGICKTGDCGGLLRCKRFGRPPTTLAEFSLNQYGKDYIDISNIKGFNVPMNFSPTTRG
CRGVRCAADIVGQCPAKLKAPGGGCNDACTVFQTSEYCCTTGKCGPTEYSRFFKRLCPDA
FSYVLDKPTTVTCPGSSNYRVTFCPTA
```

Thaumatin II (207 aa)

```
ATFEIVNRCSYTVWAAASKGDAALDAGGRQLNSGESWTINVEPGTKGGKIWARTDCYFDD
SGRGICRTGDCGGLLQCKRFGRPPTTLAEFSLNQYGKDYIDISNIKGFNVPMDFSPTTRG
CRGVRCAADIVGQCPAKLKAPGGGCNDACTVFQTSEYCCTTGKCGPTEYSRFFKRLCPDA
FSYVLDKPTTVTCPGSSNYRVTFCPTA
```

Results

General sequence searches: Thaumatin I and II amino acid full-length sequence search

Complete amino acid sequences (FASTA) for thaumatin I or II using the AllergenOnline database (January 18, 2017 version) yielded 481,602 residues in 2,035 sequences. **Thaumatin I** was found to have sequences in common with approximately 35 known allergenic proteins. The same number of "hits" (35) for known allergens was found in a full-length search for **Thaumatin II**. This is not surprising, since the two thaumatins share all but 5 amino acids in their sequence. The vast majority of hits were for thaumatin-like proteins ("TLP"), which are widely distributed across many genera. It is thought that TLP serve a protective function in the plants that synthesize them, including protection from fungal attack and/or stress; TLP may also be involved in fruit ripening (Barre 2000; Liu 2010).

The only notable difference in the results of these searches is that only thaumatin II, but not thaumatin I, contained a sequence in common to allergen from a storage mite (*Glycyphagus domesticus*); however, the similarity and identity scores between thaumatin II and storage mite allergen were moderate (0.297 and 0.514, respectively), which typically indicate poor cross reactivity between putative allergens. Importantly, the similarity does not appear in more specific searches at the 80-mer or 8-mer exact match level, suggesting only a distal relationship between the proteins.

Plant species in which thaumatin I- and II-like TLP are found include banana (*Musa acuminata*), olive (*Olea europaea*), chicle (*Manilkara zapota*), kiwi (*Actinidia deliciosa*), bell pepper (*Capsicum annuum*), Japanese cedar (*Cryptomeria japonica*), juniper (*Juniperus rigida*), cypress (*Cupressus sempervirens*), wheat (*Triticum aestivum*), sweet cherry (*Prunus avium*), peach (*Prunus persica*), apple (*Malus domestica*), and almond (*Prunus dulcis*). Many of these are known or putative allergens, or allergen precursors. Some plants (e.g. apple and banana) have multiple entries for similar thaumatin-like proteins.

Thaumatins show amino acid sequence similarities of $\geq$80% to TLP found in banana, olive, cichle and kiwi, and similarities of 65-79% to TLP found in bell pepper, wheat, cherry, peach, almond, and several conifers including cedar, juniper and cypress. These findings are not surprising since thaumatins appear to be highly conserved across genera. The presence of TLP in such a wide range of domesticated dicot and monocot food species underscores the multiple sources of TLP to which humans are exposed in their normal diet.

Specific searches

Thaumatin I sliding 80-mer window search. When searching over a sliding 80-mer window for aa similarity for **Thaumatin I**, 128 80-mers were found representing 33 sequences with some identity. The 33 hits were for TLP belonging to the same plant species identified in the full-length search. The identities of the hits to thaumatin I ranged from a low of 41.8% (peach) to 64.2% (kiwi), with an exceptionally high identity of 71.3% to banana fruit TLP.

Thaumatin I 8-mer exact match sequence search. The same search for **Thaumatin I** similarities but restricted to 8-aa exact sequence matches revealed 15 matches to allergenic proteins from 2 species: These were:

a. Chain A of banana (*Musa acuminata*) thaumatin-like antifungal protein (10 peptides);
b. Ole e 13 thaumatin-like protein precursor from banana (*Musa acuminata;* 1 peptide);
c. β-1,3-glucanase thaumatin-like protein from banana fruit (*Musa acuminata;* 2 peptides); and
d. PR5 thaumatin-like allergen precursor from juniper (*Juniperus rigida;* 2 peptides);

Thaumatin II sliding 80-mer window search. When searching over a sliding 80-mer window for aa similarity for **Thaumatin II**, as with Thaumatin I, 128 80-mers were also found (the two proteins have the same number of amino acids), corresponding to 33 sequences with some identity. The 33 hits were for TLP belonging to the same plant species identified in the full-length search. The identities of the hits to thaumatin II ranged from a low of 41.8% (peach) to 65.0% (chicle), with an exceptionally high identity of 72.5% to banana fruit TLP.

Thaumatin II 8-mer exact match sequence search. The same search for **Thaumatin II** similarities but restricted to 8-aa exact sequence matches revealed 17 peptide matches to allergenic proteins from only one species, banana:

a. Chain A of banana (*Musa acuminata*) thaumatin-like antifungal protein (14 peptides);
b. Ole e 13 thaumatin-like protein precursor from banana (*Musa acuminata;* 1 peptide); and
c. β-1,3-glucanase thaumatin-like protein from banana fruit (*Musa acuminata;* 2 peptides).

Summary

Table 6-1 summarizes the results of amino acid sequence analyses performed *in silico*.

The most closely matched amino acid sequences to *Thaumatococcus* thaumatin I and II proteins are found in TLP in banana fruit (*Musa acuminata*). Of further interest is the finding that thaumatin-like proteins, once

thought to be present only in dicots, also appear in the fruits of monocots (e.g. banana) and may serve a similar anti-fungal or other protective function (Barre 2000; Liu 2010).

Table 6-1. Bioinformatic amino acid scan for allergenic sequences in thaumatins

Thaumatin	>35% allergen similarity at indicated search granularity			TLP Species Prevalence	Allergenicity Potential
	Full seq	80-mer	8-mer		
I	35 identified	33 similar seq	15 matches	Banana (13/15)	Low
II	35 identified	33 similar seq	17 matches	Banana (17/17)	Low

For perspective, TLP are polypeptides of about 200 amino acid residues that share sequence similarity with thaumatins from *Thaumatococcus daniellii*. Due to their inducible expression by stresses like pathogen/pest attack, plant TLP are classified as the pathogenesis-related (PR) protein family 5 (PR5), which is one of 17 families of defense-related PR proteins (Christensen 2002; van Loon 2006).

A review of plant TLP by Velazhahan et al. (1999) described the occurrence, properties, and regulation of plant PR5 genes. Over the past two decades, TLP have been discovered in a wide range of organisms (Shatters 2006), including nematodes (Kitajima 1999), insects (Brandazza 2004), fungi (Grenier 2000; Sakamoto 2006), and both gymnosperms (Midoro-Horiuti 2000; Piggott 2004; Zamani 2004; Futamura 2006; O'Leary 2007; Liu 2010) and angiosperms (Velazhahan 1999; van Loon 2006).

In plants, TLP are not confined to vegetative tissues; they are also found in various fruits. For example, cherries, tomatoes and grapes accumulate large quantities of PR-5 proteins during ripening (Vu 1994; Fils-Lycaon 1996; Tattersall 1997). Hence, these fruit-specific PR-5 proteins may be involved in fruit ripening, in addition to conferring protection against fungal attack. Barre (2000) isolated and characterized TLP that accumulate in large quantities in ripening banana fruits together with other proteins involved in starch metabolism, senescence and defense (e.g. chitinase, β-1,3-glucanase). A very similar protein was also found in plantains.

In bananas (*Musa acuminata*), Barre (2000) found that the edible part of the plant (i.e. the fruit) contained between 50 and 250 mg TLP per kg of fruit flesh; that's 50-250 ppm (w/w). As a popular food, bananas can be consumed in unlimited quantities and are not associated with a high allergenic potential, even though very high levels of several thaumatin-like proteins are found in the fruit.

The published literature show that TLP are consumed from multiple sources in our daily diet. Like any foreign protein, they have the potential for allergenicity. Yet, the lack of literature linking TLP to severe food allergies or anaphylactic reactions suggests that TLP, including thaumatins, present a low allergenic risk relative to other allergens in common foods, such as peanut, shellfish or gluten.

Allergenicity of *Thaumatococcus* thaumatins as determined in preclinical and clinical studies

Higginbotham et al. (1983) performed detailed acute and sub-chronic toxicological evaluations of *Thaumatococcus*-extracted thaumatins, including determining the allergenic and anaphylactic potential of these proteins in rodents, NHP (non-human primates), and human volunteers. Their multi-species studies were comprehensive, and included evaluating the potential toxicity of purified thaumatin proteins and plant-

derived impurities from *Thaumatococcus,* as well as formulation excipients. For example, they compared the safety of purified thaumatins I and II (95-98% protein) to that of less pure (78-85%) formulations. Their studies showed no evidence of thaumatin-related toxicity. Their key findings with respect to allergenicity are summarized.

Results

Allergenic potency. Because thaumatins are foreign proteins they were evaluated for allergenic potency. Thaumatin formulations were examined for their ability to induce sensitization in guinea-pigs, anaphylactic antibody response in rats, and non-immunological histamine release *in vivo* (baboon model) and *in vitro* (from rat mast cells). In the first study, groups of 5 Guinea pigs each were injected intramuscularly with thaumatins or egg albumin (50 mg in 0.1 mL) together with a potent adjuvant (Freund's complete). Animals were sacrificed at day 12 or day 30. Sections of ileum were challenged *ex vivo* with the relevant protein antigen to measure IgE-mediated contractility.

In the second study, groups of 5 male rats each were sensitized subcutaneously with either thaumatins or egg albumin at four separate sites, using 10 micrograms of each protein per site. Animals were sacrificed at day 11 or 12 and the level of anaphylactic antibodies were compared.

In a third study, mast cells were recovered from control rats and incubated with thaumatins or egg albumin to assess histamine release. Additionally, a range of concentrations of thaumatins (0.1 ml) were injected into duplicate sites of the shaved abdomen of a baboon immediately after an intravenous injection of indicator dye. The area of 'blueing' was measured and compared with the responses obtained with isotonic saline or a synthetic peptide control.

The results of these allergenicity studies uniformly showed that thaumatins are weak sensitizers, comparable to egg albumin, if administered systemically but not if administered orally.

Human clinical and occupational exposure studies. Occupational exposure and oral ingestion studies with humans were conducted to assess the allergenic potential of various thaumatin-containing substances. Higginbotham et al. (1983) evaluated 140 personnel who worked at a thaumatin-extraction facility in the UK and were exposed to dust containing thaumatin for a period of up to 7 years.

Several thaumatin-containing substances were tested using the skin prick test. These included commercial thaumatin, purified thaumatin I, purified thaumatin II, freeze-dried thaumatin gum arabic mixture, non-thaumatin plant constituents (i.e. host-derived impurities, and spray-dried thaumatin gum arabic mixture). These substances represented what workers at the factory had inhaled and contacted during the multi-year period of exposure.

Prick testing of laboratory personnel who had been intermittently exposed by inhalation to thaumatin in their work environment showed that 9.3% (13/140) responded positively to commercial thaumatin, while 30.7% were positive to *Dermatophagoides pteronyssinus* (house dust mite). Challenge tests in man did not demonstrate any oral sensitization. Allergic responses to purified thaumatins were comparable to responses to commercial (less pure) thaumatin.

A second clinical study was conducted in which 10 volunteers (6 men and 4 women) ingested gelatin capsules containing thaumatin or lactose (100 mg/dose) daily for 14 days, to assess the potential for oral sensitization in humans. The initial exposure was followed by an additional cross-over period of 14-days. Administration of capsules and evaluation of results was done in a double-blind fashion to minimize bias. Wheal and erythema and serology were used to assess sensitization. Sera separated from the blood of volunteers

participating in the oral-challenge capsule study showed no anaphylactic antibodies to thaumatin. Additionally, no sensitization was detected in prick tests at the end of the study.

Summary

Extensive preclinical and two human clinical studies verified the low allergenicity of thaumatins. Some commercial thaumatins from *Thaumatococcus* available even today may contain potentially allergenic and/or toxic impurities, including proteases, thaumatin variants and thaumatin-like proteins (some of which, as discussed above, are known allergens), aluminum salts and other impurities and contaminants (Asherie 2008; Nikolic 2014).

In contrast, Notifier's THAUMATIN is devoid of impurities and contaminants associated with *Thaumatococcus* extraction, and any residual host-derived proteins or other host components are found in low concentration (see APPENDIX C THAUMATIN Characterization for detail).

6.3. Safety in Relation to Dietary Intake of THAUMATIN

We expect the same levels of consumption of Notifier's thaumatins per weight or volume of treated food as those allowed for extracted thaumatins. EFSA provides guidance on maximum permitted levels (MPL) of thaumatin on various foods and beverages according to Annex II to Regulation (EC) No 1333/2008 (OJEU 2011). Table 6-2 lists the authorized **original and expanded-use levels for thaumatin (E 957)** in mg/kg food (w/w) and/or mg/L for beverages or non-solid foods (w/v).

Although it excludes sweetener applications, FEMA's GRAS List (Cohen 2016) provides application rates for thaumatin in the USA. Table 6-3 lists the **original and expanded-use levels of extracted and recombinantly produced thaumatins** for US food products.

Thaumatin's EU food additive designation number is E 957. The MPL for E 957 ranges from a low of 5 mg/kg in "flavored fermented milk products" (e.g. yogurt) to a high of 400 mg/kg in "food supplements supplied in a syrup-type or chewable form" (EFSA 2015). The same regulations allow the unlimited addition of thaumatin (*Quantum satis*) when the protein is used as a table top sweetener. It is envisioned that THAUMATIN could be used at equivalent application rates on various foods.

The application rates of thaumatin to various food products (maximum permissible level, MPL) were derived by assessing the consumption of each food type and estimating the additive intake to reach a maximum exposure of 1.1 mg thaumatin/kg body weight (bw) per day, or 77 mg/day for a 70-kg person**.** Consumption values were derived from typical European diets, taking into consideration country specific dietary preferences as well as various population age groups (**infant, adult, elderly**).

The original application levels allowed by EFSA for a limited number of foods in the EU were first published in 1988, and later amended (expanded) in 2011 to include more food types and higher allowed levels of thaumatin (see OJEU 2011 for detail). Revision of statutes for expanded use of thaumatin was based on thaumatin's functionality and post-market safety record.

In surveying the types of sweeteners applied to foods, and the consumption statistics for those foods in typical US diets (derived from USDA sources, including Haley 2011 and McConnell 2016), it was concluded that US and Western European diets were not sufficiently different to warrant separate calculations of consumption. Hence, the same consumption assumptions and limits found in extensive studies in the EU are expected to apply to domestic (US) consumption of thaumatin.

Table 6-2. EFSA Currently authorized and extended use levels (2015) for thaumatin (E 957)

FCS category number	Food categories	Currently authorised MPL (mg/L or mg/kg as appropriate)	Currently authorised and change of use levels and proposed extension of use (mg/L or mg/kg as appropriate)	Restrictions/ exception
1.4	Flavoured fermented milk products including heat-treated products	5	5	Only as a flavour enhancer
3	Edible ices	50	50	Only energy reduced or with no added sugar
4.2.5	Jams, jellies and marmalades and similar products		5	Energy reduced or with no added sugar
5.1	Cocoa and chocolate products as covered by Directive 2000/36/EC	50	50	Only energy reduced or with no added sugar
5.2	Other confectionery including breath refreshening microsweets	50	50	Only cocoa or dried fruit based, energy reduced or with no added sugar
5.2	Other confectionery including breath refreshening microsweets	50	50	Only confectionery with no added sugar
5.3	Chewing gum	10	10	Only with added sugar or polyols, as flavour enhancer
5.3	Chewing gum	50	50	Only with no added sugar
5.4	Decorations, coatings and fillings, except fruit-based fillings covered by category 4.2.4	50	50	Only confectionery with no added sugar
5.4	Decorations, coatings and fillings, except fruit-based fillings covered by category 4.2.4	50	50	Only cocoa or dried fruit-based, energy-reduced or with no added sugar
6.3	Breakfast cereals		10	Energy reduced or with no added sugar
7.2	Fine bakery wares		30	
11.4.1	Table-top sweeteners in liquid form	*Quantum satis*[a]	*Quantum satis*[a]	
11.4.2	Table-top sweeteners in powder form	*Quantum satis*[a]	*Quantum satis*[a]	
11.4.3	Table-top sweeteners in tablets form	*Quantum satis*[a]	*Quantum satis*[a]	
12.1.2	Salt substitutes		15	
12.5	Soups and broths		15	
12.6	Sauces		15	
14.1.4	Flavoured drinks	0.5	5	Only water-based flavoured non-alcoholic drinks. As flavour enhancer only
14.2.1	Beer and malt beverages		5	
14.2.2	Wine and other products defined by Regulation (EEC) No 1234/2007 and alcohol-free counterparts		5	
14.2.4	Fruit wine and made wine		5	
14.2.6	Spirit drinks as defined in Regulation (EC) No 110/2008		5	
14.2.7	Aromatised wine-based products as defined by Regulation (EEC) No 1601/91		5	
14.2.8	Other alcoholic drinks including mixtures of alcoholic drinks with non-alcoholic drinks and spirits with less than 15 % of alcohol		5	
15.1	Potato-, cereal-, flour- or starch-based snacks		10	
16	Desserts excluding products covered in category 1, 3 and 4	5	5	As flavour enhancer only
17.1	Food supplements supplied in a solid form, including capsules and tablets and similar forms excluding chewable forms		400	
17.2	Food supplements supplied in a liquid form		400	
17.3	Food supplements supplied in a syrup-type or chewable form	400	400	
18	Processed foods not covered by categories 1 to 17, excluding foods for infants and young children		10	Ready meals (chilled, frozen, dried) only

FCS: Food Categorisation System.
(a): Although permitted at *quantum satis* levels, a level of use of 10 mg/kg in table-top sweeteners (liquid, powder and tablet form) is considered an appropriate current level of use by the applicant and was used in the intake assessment.

Table 6-3. FEMA Updated thaumatin use levels (2016)

	Thaumatin	Neohesperidine dihydrochalcone	Thaumatin B-Recombinant	N-(2-Methylcyclohexyl)-2,3,4,5,6-pentafluorobenzamide	Glucosyl steviol glycosides
Category/FEMA No.	3732	3811	3814	4678	4728
Baked Goods	7*/7*	5/7*	1/1	1*/5*	150*/500*
Beverages, Non-Alcoholic	7*/7*	5/15	5/7*	1/5	125/175*
Beverages, Alcoholic	7*/7*	5/15	5/7*	0/0	125/175*
Breakfast Cereals	7*/7*	8/20	1/2	0/0	200*/500*
Cheeses	7*/7*	3/4	7*/7*	0/0	100/133*
Chewing Gum	150*/150*	200/300	150/150*	10/20	500*/1500*
Condiments and Relishes	7*/7*	2/3	1/2	0/0	125/200*
Confections and Frostings	7*/7*	3/3	2/5	1/5	50/100
Egg Products	7/7*	2/3	2/5	0/0	0/0
Fats and Oils	7*/7*	4/4	0/0	0/0	125/189*
Fish Products	7*/7*	2/3	5/7*	0/0	0/0
Frozen Dairy	7/7*	2/8	1/2	1/5	125/133*
Fruit Ices	7/7*	2/3	2/5	1/5	125/133*
Gelatins and Puddings	7*/7*	3/8	1/2	1/5	125/133*
Granulated Sugar	0/0	0/0	0/0	0/0	0/0
Gravies	7*/7*	3/4	2/5	0/0	125/133*
Hard Candy	7*/7*	5/15	2/5	1/5	100/133*
Imitation Dairy	7*/7*	3/10	7*/7*	0/0	125/250*
Instant Coffee and Tea	7*/7*	3/6	2/5	1/5	125/175*
Jams and Jellies	7*/7*	2/3	2/5	1*/5*	125*/200*
Meat Products	7*/7*	2/3	2/2	0/0	0/0
Milk Products	7*/7*	3/10	3/6	1/5	133*/225*
Nut Products	7*/7*	3/4	5/7*	0/0	133*/175*
Other Grains	7*/7*	3/4	0/0	0/0	100/133*
Poultry	7*/7*	2/3	2/5	0/0	0/0
Processed Fruits	7*/7*	2/3	2/5	1/5	133*/200*
Processed Vegetables	7*/7*	2/3	2/5	0/0	100/133*
Reconstituted Vegetables	7*/7*	2/3	2/5	0/0	133*/133*
Seasonings and Flavors	7/7*	3/4	0.5/1	0/0	133*/175*
Snack Foods	7*/7*	3/3	1/2	0/0	133*/133*
Soft Candy	7*/7*	4/10	2/5	1/5	100/133*
Soups	7*/7*	5/7*	2/5	0/0	133*/133*
Sugar Substitutes	0/0	0*/0*	0*/0*	0/0	0*/0*
Sweet Sauces	7*/7*	2/3	2/5	1/5	133*/133*

Interim FEMA GRAS™ 28 List (Aug 2016). Columns 1 and 3, extracted and recombinant thaumatins, respectively. FEMA Updated average usual use levels (ppm)/average maximum use levels (ppm) for flavoring substances previously recognized as FEMA GRAS. Superscript (*) represents a new use level (Cohen 2016).

While near-term THAUMATIN dietary intake levels are easier to project based on currently allowed application rates in many countries, future trends are more difficult to forecast and are the topic of several recent studies. Ng et al. (2012) tried to quantify the use of caloric and non-caloric sweeteners in US consumer packaged foods. They reported that of the 85,451 uniquely formulated foods purchased during 2005–2009, 75% contained sweeteners (68% with caloric sweeteners (CS) only, 1% with non-caloric sweeteners (NCS) only, 6% with both CS and NCS). CS were used in >95% of cakes/cookies/pies, granola/protein/energy bars, ready-to-eat cereals, sweet snacks, and sugar-sweetened beverages. NCS were in >33% of yogurts and sports/energy drinks, 42% of waters (plain or flavored), and most diet sweetened beverages.

Across unique products, corn syrup was found to be the most commonly listed sweetener, followed by sorghum, cane sugar, high fructose corn syrup and fruit juice concentrate (FJC). Ng et al. also reported that 77% of all calories purchased in the US in 2005–2009 contained CS and 3% contained NCS, while 73% of the volume of foods purchased contained CS and 15% contained NCS. Trends during this period suggest a shift towards the purchase of NCS-containing products.

More recently, a survey by Sylvetsky et al. (2017) that tracked US consumption of low-calorie sweeteners (LCS) showed that intake has increased significantly during the past several decades. The goal of the Sylvetsky et al. study was to describe LCS consumption in the United States and to characterize consumption by sociodemographic subgroups, source, frequency, eating occasion, and location. The sources of information were the National Health and Nutrition Examination Survey (**NHANES**) data from 2009-2010 and 2011-2012. Sylvetsky et al. found that total of 4,981 unique food and beverage items were consumed by participants in NHANES 2009-2010. Of these items, 126 contained LCS, including 57 beverages, 61 foods, and 8 packets. Similarly, 5,192 unique food and beverage items were reported in NHANES 2011-2012. Of these items, 147 contained LCS, including 74 beverages, 65 foods, and 8 packets.

Importantly, Sylvetsky et al. were not able to quantify the amount of LCS in LCS-containing products because manufacturers are not required to provide information regarding the quantity of LCS added, with the exception of saccharin. Due to the inability to quantify the amount of LCS in foods and beverages, intake (in grams) of LCS-containing products was estimated as a proportion of an individual's total intake of the specific product category (eg, yogurts, desserts) reported in NHANES (Sylvetsky 2017).

The food categories in NHANES surveyed by Sylvetsky (2017) are very similar to the categories surveyed in the EU, with the possible exception that serving portions of some foods tend to be larger in the USA than they are in Europe; nevertheless, the EFSA application rates provide an accurate basis for estimating potential US intake of thaumatins based on the types of foods consumed. Therefore, Notifier assumed a "highest use" scenario for intake of thaumatin in the diet, and derived use levels from the broadest groups of foods that could be treated. Not coincidentally, the EFSA/EU database and use levels by food group presented the most comprehensive source of information, because thaumatin is already approved for such uses in the EU.

To summarize, a preliminary but accurate assessment of the potential dietary intake of thaumatin can best be derived from EU usage data, which show that, from all dietary sources, continuous thaumatin ingestion levels of up to 1.1 mg/kg (bw)-day, or 77 mg/person-day, are safe. The commercial introduction of Notifier's THAUMATIN product is expected to displace, initially, thaumatin that is currently derived from extraction of the same proteins from *Thaumatococcus*. Should industrial thaumatin usage expand due to availability of THAUMATIN, consumption of thaumatin would be expected to increase. Even if daily per capita intake were to double to 150-200 mg from the current 77 mg, thaumatin's safety factor of 1,300 based on chronic toxicity studies suggest that any additional safety risk to consumers would be minimal (Hagiwara 2005; OJEU 2011; EFSA 2015). As a sweetener, thaumatin can be used *Quantum satis* because of its very high safety margin (EFSA 2015).

6.4. Occupational safety

With respect to occupational safety, Notifier's thaumatin proteins are considered non-toxic. No higher-level precautions for personnel protection are suggested for handling, preparing or disposing of THAUMATIN powder or liquid solutions. Only general precautions for handling powdered bulk substances and protein-containing waste solutions are suggested. A SDS for THAUMATIN is appended to this Notice (APPENDIX A), where additional recommendations for storage, handling and disposal of this product are listed.

6.5. Overall safety summary in support of THAUMATIN's GRAS designation

The results of clinical studies by Higginbotham et al. (1983) assessing the effects of occupational pulmonary exposure to thaumatin-containing substances, as well as the effects of oral ingestion of thaumatins, corroborate the results they obtained with multiple animal species that show that thaumatin is digestible, shows no systemic toxicity when administered orally, and has low allergenic potential if delivered systemically but none if delivered orally.

The sub-acute (28-day with 14-day thaumatin plus 14-day lactose cross-over) ingestion study with human volunteers administered a total of 1.4 grams of thaumatin over a two-week period, with no ill effects. Similarly, the multi-year occupational exposure to low levels of thaumatin-containing dust in the work place had no obvious toxic effects and only low measurable sensitivity, which was lower in prevalence than allergy to dust mites in the surveyed population.

Subsequently, Hagiwara et al. (2005) reported results of a 13-week (sub-chronic) feeding study of thaumatin in Sprague–Dawley rats. Thaumatin was administered at dietary levels of 0% (control), 0.3%, 1.0% and 3.0% to groups of 10 male and 10 female Crj:CD (SD) IGS rats. There were no treatment-related clinical signs or adverse effects on the survival rate, body weight, food consumption, water consumption and urinalysis, ophthalmology, hematology, or blood biochemistry data. No treatment-related alterations in gross pathology or organ weights were found in any group. On histopathological examination, sporadic spontaneous lesions known to occur in this strain of rats were the only findings, with no specific relation to the test substance.

Hagiwara et al. (2005) calculated the no-observed-adverse-effect-level (NOAEL) to be a dietary intake of at least 3.0% (2,502 mg/kg body weight/day for males, 2,889 mg/kg body weight/day for females) under the experimental conditions used. Studies in dogs established a lower average NOAEL of 1,400 mg/kg body weight/day. These studies helped support an extensive safety review conducted by the joint WHO/FAO Expert Committee on Food Additives, the EU Scientific Committee on Food (SCF 1985, 1985) and by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 1989. The levels of usage of thaumatin on various food groups, the consumption of each food type, and the anticipated total exposure to thaumatin as a food additive have since been aggregated and updated (EFSA 2015).

Based on the lowest NOAEL of 1,400 mg/kg bw-day for dogs calculated from results by Higginbotham et al. (1983) and Hagiwara et al. (2005) and others, EFSA applied a **safety factor of ~1,300 for E 957** (1,400 mg/kg bw-day in dogs and the then-current human maximum daily intake level of 1.03 mg/kg body wt-day) to arrive at an MPL for thaumatin by food product group, including solids, semi-solids and liquid beverages, for all age groups. The cumulative daily limit for chronic human consumption was set at 1.1 mg/kg-day, or 77 mg/day for a 70-kg person. At this intake level, thaumatin protein would represent <0.13% of the total adult daily protein intake (i.e. nutritionally insignificant). Even at 2x the current intake level, the safety factor would be nearly 700:1.

The safety of Notifier's THAUMATIN product can be deduced by analogy to the thaumatin products evaluated in the studies cited herein. Higginbotham et al. (1983) evaluated thaumatin compositions of various degrees

of purity, including commercial thaumatin (Thalin®) that contained 3% residual host impurities, including non-thaumatin proteins, carbohydrates, pectin, and glucuronoxylan from the arils of the source fruit. Ramsohoye (1989) found non-thaumatin protease and esterase impurities in commercial thaumatin products. In addition, because the original Tate & Lyle thaumatin extraction process involved the use of aluminum salts to assist with purification, the final product contained small amounts of the heavy metal. Importantly, in spite of these findings, the toxicity studies of Higginbotham et al. included evaluation of isolated host and process impurities, which proved to be innocuous with respect to safety.

Taken in context, Notifier's product has no higher level of plant host impurities than the current commercial products, and the thaumatin flavor modifying active ingredients, thaumatins I and II, are identical to those of the extracted thaumatins. Hence, Notifier expects that the high safety margin calculated for extracted thaumatins and reported by others in the peer-reviewed public literature will also apply to the safety of its recombinantly produced thaumatin proteins. Given the very low levels of thaumatins that are applied to foods, residual constituents in the product that are not thaumatin would be at such low levels (2-3% w/w of total thaumatin protein) as to present, at worst, a minimal safety risk.

7. Supporting Data and Information

Multiple sources of information were used to support the conclusion that Notifier's THAUMATIN product is GRAS. Table 7-1 lists the various data and other information discussed in this Notice and used in reaching this conclusion. Also listed in the table is whether the specific information cited was generated by Notifier and/or obtained from databases or references in the public domain.

The active ingredients (thaumatin I and thaumatin II) in Notifier's THAUMATIN product are of the same molecular composition as the active ingredients in thaumatin-containing products derived from extracts of *Thaumatococcus* and have the same functional properties. Because Notifier's formulation is no less pure than commercially available thaumatins currently allowed for food use, we draw analogies to those referenced products with respect to flavor-modifying functionality, dietary use levels, and safety.

Table 7-1. Information supporting THAUMATIN GRAS determination

Topic	Location in this GRN	Source of data or information	Availability
Thaumatin identity	Section 2.1	Inglett 1968; Suami 1997; van der Wel 1972; Lee 1987; Hough 1993; Zemanek 1995; Greenly 2003	Public
Structure & function	Section 2.1	Suami 1997; de Vos 1985; Ogata 1992; van der Wel 1984; Hough 1993; Faus 2008	Public
Mode of action and mechanisms of taste modification	Section 2.1	Bartoszewski 2003; Faus 2008; Glaser 2000; Greenly 2003; Hellekant 1996; Kaneko 2001; Kinghorn 1986; Ledeboer 1984; Lim 2012; Masuda 2016; Nagarajan 2017; Ohta 2011; Sardesai 1991; Schiffman 1995; Suami 1997; Tinti 2000; van der Wel 1972; Witty 1990	Public
Method of manufacture & QC	Section 2.2; APPENDIX B	Nomad Bioscience GmbH	This GRN
Host, vector and process impurities	Section 2.2; APPENDIX B	Nomad Bioscience GmbH	GRN 593 and This GRN

Topic	Location in this GRN	Source of data or information	Availability
Composition & specification	Section 2.3	Nomad Bioscience GmbH	This GRN
Allowed dietary exposure; estimated dietary intake, and additional exposure not related to Notifier's product	Sections 3.1 & 3.2	OJEU 2011; EFSA 2008; EFSA 2011; EFSA 2015; Cohen 2016; Codex Alimentarius 2016; Nomad Bioscience GmbH	Public and This GRN
Thaumatin overall safety; digestibility; potential for gene transfer to intestinal microflora	Section 6.1	JECFA 1983, 1986; EFSA 2015; SCF 1985, 1989; EFSA 2011; Higginbotham 1983; Hagiwara 2005; Szwacka 2012	Public
Estimation of thaumatin's NOAEL	Section 6.1	Higginbotham 1983; Hagiwara 2005	Public
Determination of thaumatin's allergenic potential	Section 6.2	a. Computational estimates / *in silico* modeling: Baniulis 2008; Nomad Bioscience GmbH b. Empirical studies, preclinical and clinical Higginbotham 1983	a. Public and This GRN b. Public
Commonly consumed and environmentally contacted thaumatin-like proteins (TLP)	Section 6.2	Barre 2000; Liu 2010; Christensen 2002; van Loon 2006; Velazhahan 1999; Shatters 2006; Kitajima 1999; Brandazza 2004; Grenier 2000; Sakamoto 2006; Midoro-Horiuti 2000; Piggott 2004; Zamani 2004; Futamura 2006; O'Leary 2007; Vu 1994; Fils-Lycaon 1996; Tattersall 1997	Public
Allergenicity and occupational safety	Section 6.2	Higginbotham 1983	Public
Safety in relation to allowed dietary intake of thaumatin	Section 6.3	EFSA 2015; OJEU 2011; Codex Alimentarius 2016; Oser 1984; Smith 1996; Cohen 2016	Public
Potential additional exposure if patterns of consumption of low/no calorie sweeteners increase	Section 6.3	NHANES 2009-10, 2011-12 in Sylvetsky 2017; USDA ERS in Haley 2011, McConnell 2016; Ng 2012	Public
THAUMATIN Safety Data Sheet (SDS)	APPENDIX A	a. Nomad Bioscience GmbH b. Sigma-Aldrich 2017; SFC 2006; SCBT 2015	a. This GRN b. Public
Thaumatin analysis and characterization	APPENDIX C	Nomad Bioscience GmbH	This GRN

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APPENDIX A. THAUMATIN Safety Data Sheet

NOMAD

DRAFT– THAUMATIN SAFETY DATA SHEET
Version 1.0
Revision Date 10/01/2017
Print Date 10/01/2017

1. PRODUCT AND COMPANY IDENTIFICATION

1.1 Product identifiers

Product name	:	THAUMATIN from plants (e.g. lettuce, red beet, spinach)
	:	CAS Number 53850-34-3 (extracted); CAS Number 553850-34-3 (recombinant)
	:	Consists of thaumatin I, thaumatin II, or a blend of thaumatins I and II, of identical amino acid sequence to thaumatins from *Thaumatococcus daniellii*

1.2 Functional uses

Identified uses	:	Sweetener, flavor modifier/enhancer, for use in food or feed

1.3 Manufacturer

Company	:	Nomad Bioscience GmbH Weinbergweg 22 Halle 02160, Germany
Telephone	:	+49 345 555 9887
Fax	:	+49 345 1314 2601

1.4 Emergency telephone number

Emergency Phone Number	:	+49 345 555 9887 in the EU (US emergency phone number to be provided)

2. HAZARDS IDENTIFICATION

2.1 Classification of the substance or mixture
Not a hazardous substance or mixture

2.2 GHS Label elements, including precautionary statements
Not a hazardous substance or mixture

2.3 Hazards not otherwise classified (HNOC) or not covered by GHS
None

2.4 HMIS Classification

Health hazard:	0
Flammability:	0
Physical hazards:	0

2.5 NFPA Rating

Health hazard:	0
Fire:	0
Reactivity hazard:	0

2.6 Potential Health Effects of Bulk Powder/Concentrate

Inhalation	May be harmful if inhaled. May cause respiratory tract irritation
Skin	May be harmful if absorbed through skin. May cause skin irritation
Eyes	May cause eye irritation
Ingestion	May be harmful if swallowed

3. COMPOSITION/INFORMATION ON INGREDIENTS

3.1 Substances

CAS Registry Number	:	53850-34-3 (extracted); 553850-34-3 (recombinant)

No ingredients are hazardous according to OSHA criteria
No components need to be disclosed according to the applicable regulations

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4. FIRST AID MEASURES

4.1 Description of first aid measures

If inhaled

If breathed in, move person into fresh air. If not breathing, give artificial respiration

In case of skin contact

Wash off with soap and plenty of water

In case of eye contact

Flush eyes with water as a precaution

If swallowed

Never give anything by mouth to an unconscious person. Rinse mouth with water

5. FIREFIGHTING MEASURES

5.1 Extinguishing media - Suitable extinguishing media

Use water spray, alcohol-resistant foam, dry chemical or carbon dioxide

5.2 Hazardous combustion products

Hazardous decomposition products formed under fire conditions – Carbon oxides

5.3 Advice for firefighters

Wear self-contained breathing apparatus for firefighting if necessary

5.4 Additional information

No additional information is available

6. ACCIDENTAL RELEASE MEASURES

6.1 Personal precautions, protective equipment and emergency procedures

As with any concentrated protein, avoid dust formation and inhalation of particulates or aerosols. For personal protection see Section 8

6.2 Environmental precautions

Product active ingredients are biodegradable. No special environmental precautions are necessary

6.3 Methods and materials for containment and cleaning up

Sweep up and shovel solid. Water-wash surfaces. Use closed containers for disposal of any unused product

6.4 Reference to other sections

For disposal see Section 13

7. HANDLING AND STORAGE

7.1 Precautions for safe handling

Provide appropriate exhaust ventilation during preparation and use. Provide appropriate exhaust ventilation at places where dust is formed. Normal measures for preventive fire protection

7.2 Conditions for safe storage, including any incompatibilities

Keep container tightly closed in a dry well-ventilated place. Recommended storage temperature 2-8 °C

8. EXPOSURE CONTROLS/PERSONAL PROTECTION

8.1 Control parameters

Components with workplace control parameters

Contains no substances with occupational exposure limit values

8.2 Exposure controls

Appropriate engineering controls

General industrial hygiene practice

Personal protective equipment

Eye/face protection
Use government tested and approved eye protection devices (e.g. NIOSH - US or EN 166 - EU)

Skin protection
Handle with gloves that are inspected prior to use. Use proper glove removal technique to avoid skin contact. Dispose of used gloves in accordance with applicable laws and good laboratory practices. Wash and dry hands

Body Protection
Wear lab coat or similar cover during preparation, application and disposal of product in keeping with specific practices in the work environment

Respiratory protection
Use type N95 (US) or type P1 (EN 143) dust masks, or respirators, depending on the product formulation and preparation and use environment. Use devices approved under appropriate government standards such as NIOSH (US) or CEN (EU)

Control of environmental exposure
Product components are biodegradable and will be diluted during use. No special procedures for controlling environmental exposure are recommended

9. PHYSICAL AND CHEMICAL PROPERTIES

9.1 Information on basic physical and chemical properties

a)	Appearance form:	Solid; yellowish-white to light tan
b)	Odor	No specific odor
c)	Odor threshold	No odor threshold identified
d)	pH	pH 4-8, depending on the formulation
e)	Melting point/freezing point	No data available
f)	Initial boiling point and boiling range	No data available
g)	Flash point	No data available
h)	Evaporation rate	No data available
i)	Flammability (solid, gas)	No data available
j)	Upper/lower flammability or explosive limits	No data available
k)	Vapor pressure	No data available
l)	Vapor density	No data available
m)	Relative density	No data available
n)	Water solubility	>25 g/L
o)	Partition coefficient: n-octanol/water	No data available
p)	Auto-ignition temperature	No data available
q)	Decomposition temperature	No data available
r)	Viscosity	No data available
s)	Explosive properties	No data available
t)	Oxidizing properties	No data available

9.2 Other safety information
No additional information available

10. STABILITY AND REACTIVITY

10.1 Reactivity
No data available

10.2 Chemical stability
Stable under recommended storage conditions

10.3 Possibility of hazardous reactions
No data available

10.4 Conditions to avoid
Strong oxidizing agents

10.5 Incompatible materials
No data available

10.6 Hazardous decomposition products
Other decomposition products – Carbon oxides. In the event of fire: See Section 5

11. TOXICOLOGICAL INFORMATION

11.1 Information on toxicological effects

Acute toxicity
No data available

Inhalation
No data available

Dermal
No data available

Skin corrosion/irritation
No data available

Serious eye damage/eye irritation
No data available

Respiratory or skin sensitization
No data available

Germ cell mutagenicity
No data available

Carcinogenicity

IARC: No component of this product present at levels greater than or equal to 0.1% is identified as a probable, possible or confirmed human carcinogen by IARC

ACGIH: No component of this product present at levels greater than or equal to 0.1% is identified as a carcinogen or potential carcinogen by ACGIH

NTP: No component of this product present at levels greater than or equal to 0.1% is identified as a known or anticipated carcinogen by NTP

OSHA: No component of this product present at levels greater than or equal to 0.1% is identified as a carcinogen or potential carcinogen by OSHA

Reproductive toxicity
No data available

Specific target organ toxicity - single exposure
No data available

Specific target organ toxicity - repeated exposure
No data available

Aspiration hazard
No data available

Additional Information
RTECS: Not available. Product is not a hazardous substance or mixture

11.2 Potential health effects

Inhalation: May be harmful if inhaled. May cause respiratory tract irritation
Ingestion: May be harmful if swallowed
Skin: May be harmful if absorbed through skin. May cause skin irritation
Eyes: May cause eye irritation

12. ECOLOGICAL INFORMATION

12.1 Toxicity
No data available

12.2 Persistence and degradability
Active ingredients are destroyed by heat, acid, and by digestive and microbial enzymatic activity

12.3 Bioaccumulative potential
None anticipated

12.4 Mobility in soil
No data available

12.5 Results of PBT and vPvB assessment
PBT/vPvB assessment not available as chemical safety assessment not required/not conducted

12.6 Other adverse effects
No data available

13. DISPOSAL CONSIDERATIONS

13.1 Waste treatment methods

Product
Offer surplus and non-recyclable solutions to a licensed disposal company

Contaminated packaging
Dispose of as unused product

14. TRANSPORT INFORMATION

DOT (US)
Not dangerous goods

IMDG
Not dangerous goods

IATA
Not dangerous goods

15. REGULATORY INFORMATION

OSHA
No known OSHA hazards

SARA 302 Components
SARA 302: No chemicals in this material are subject to the reporting requirements of SARA Title III, Section 302

SARA 313 Components
SARA 313: This material does not contain any chemical components with known CAS numbers that exceed the threshold (De Minimis) reporting levels established by SARA Title III, Section 313

SARA 311/312 Hazards
No SARA Hazards

Massachusetts Right to Know Components
No components are subject to the Massachusetts Right to Know Act

Pennsylvania Right to Know Components
Thaumatins from plants. See Section 1.1

New Jersey Right to Know Components
Thaumatins from plants. See Section 1.1

California Prop. 65 Components
This product does not contain any chemicals known to State of California to cause cancer, birth defects, or any other reproductive harm

16. OTHER INFORMATION

Additional information

The information contained in this Safety Data Sheet is believed to be correct as of the time of its release. It should be used as a guide for safe handling, storage, preparation, and disposal of the product. Assessment of product safety under conditions of normal use is based on information available at the time. Because information in some categories is lacking, this SDS is not all-inclusive and is subject to periodic updates. Nomad Bioscience GmbH and its Affiliates shall not be held liable for any damage resulting from handling, use, disposal or from contact with the above product.

APPENDIX B. THAUMATIN Manufacturing Process

B.1. Introduction and Rationale

Notifier manufactures thaumatin proteins recombinantly using a plant-based process very similar to the process described in GRN 593, modified for the current proteins. This approach offers a more environmentally sustainable source of thaumatins in comparison to harvesting *Thaumatococcus* in the wild (Most 1978), and a more scalable and cost-effective manufacturing option relative to fermentation.

Briefly, in Notifier's process, leaf tissue of the food plants *Beta vulgaris* (**beet**), *Spinacia oleracea* (**spinach**), or *Lectuca sativa* (**lettuce**) can be transfected to express thaumatins by transient expression of a plant viral vector, such as tobacco mosaic virus (TMV) or potato virus X (PVX), containing the gene for a thaumatin protein. The safety of host and vector impurities was described in GRN 593. The components of the expression system and host plants are prepared independently and subsequently combined. Alternatively, thaumatins can be produced in the same host plants carrying transgenically the thaumatin gene and an ethanol-inducible promoter, with induction by dilute ethanol. After induction with either method, thaumatin protein accumulates in leaf tissues for several days. Plants are subsequently harvested and thaumatin is extracted and concentrated from the plant material. The sweetness and flavor-modifying properties of thaumatin I and thaumatin II proteins are equivalent. Hence, Notifier's THAUMATIN product may be formulated to contain either thaumatin I or thaumatin II individually, or a blend of both to a predetermined total content of thaumatin protein.

THAUMATIN contains no live biological materials that were introduced in the upstream steps of the process (e.g. when using *Agrobacterium* and viral replicons). The process is generic in that it is applicable to the expression and isolation of a wide range of proteins. A description of the process with respect to production of either thaumatin I or thaumatin II is summarized below.

B.2. Organism Used and Gene Expression Cassette

In the agroinduction method, the production organism *Agrobacterium tumefaciens* harboring binary plasmid vector comprising a TMV replicon with inserted thaumatin gene (either thaumatin I or thaumatin II) is depicted in Figure B-1. Thaumatins are natively expressed as pre-pro-proteins and Notifier has developed vectors that express pre-pro-proteins with native signal peptide (SP), as well as pro-proteins with native SP replaced by rice alpha amylase SP, or mature thaumatin protein without native SP and C-terminal peptide, co-expressed with rice SP. Comparison of protein quality and yield led to optimization of constructs and selection of pICH95105 and pICH95397; the latter is used preferentially for thaumatin manufacture.

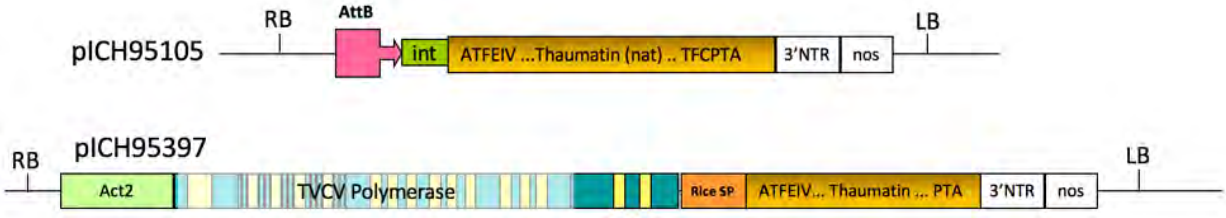

Figure B-1. *Agrobacterium* expression vector for thaumatin proteins

Mature thaumatin proteins of the correct (native) amino acid composition and sequence are efficiently expressed in agrobacterial vectors

After polypeptide processing *in planta*, the mature thaumatin protein accumulates in leaf tissue over several days post inoculation. Neither the native *Thaumatococcus* thaumatins nor the recombinant equivalents are glycosylated as there are no glycan addition sites along the backbone.

Preparation of vectors and inoculum are depicted in Figure B-2. Vectors are constructed by conventional molecular biology methods and maintained as Master and Working Plasmid Banks in *E. coli* (Figure B-2-**A**). The T-DNA vector encoding TMV-Thm replicon is transfected into *A. tumefaciens* to prepare the inoculum (Figure B-2-**B**). Each bacterium in the inoculum contains the T-DNA-TMV-Thm plasmid (Figure B-2-**C**).

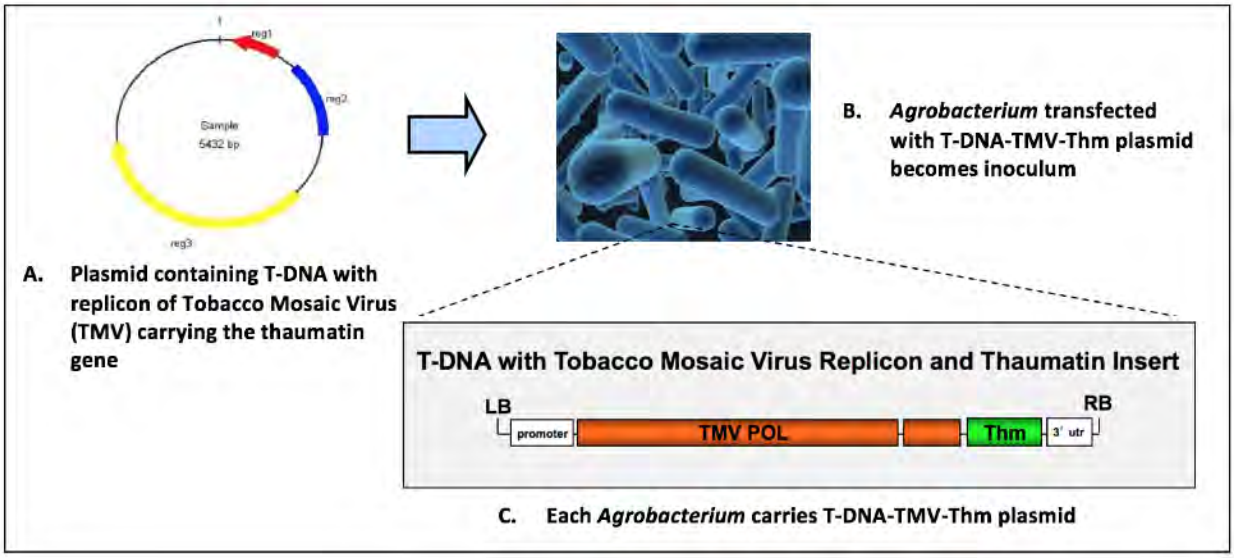

Figure B-2. Schematic of vector for thaumatin expression in plants

B.3. Procedure

A flow diagram summarizing the key steps in producing colicin proteins is shown in Figure B-3. Summary descriptions of key process steps follow; step numbers correspond to the steps indicated in Figure B-3. The induction of gene expression can be accomplished by one of two alternative methods (described below), which share common downstream purification unit operations.

Step 1a. Inoculum production for *Agrobacterium* induction method

A proprietary industrial strain of *Agrobacterium tumefaciens* harboring a binary plasmid vector containing a TMV replicon with inserted thaumatin gene (thaumatin I or II) is grown in defined medium under aseptic conditions following strict quality SOPs; this bacterial suspension constitutes the inoculum. Notifier's *Agrobacterium* strain is grown in medium containing de-mineralized water, yeast extract, peptones, minerals, kanamycin and rifampicin. The removal of residual antibiotics and fermentation chemicals is achieved by high dilution of the bacterial suspension before inoculation of plants and the ultra- and dia-filtration procedures during plant biomass extraction and processing. All raw materials and processing aids are food grade. A multi-vial Master Vector Bank of the vector is prepared and stored at -80°C, from which aliquots are removed as Working Vector Banks of the inoculum for each manufacturing batch.

Each Working Bank of *Agrobacterium* is handled in a way to reduce the risk of contamination by foreign microorganisms. This includes use of sterile materials for bacterial cultivation, quality control checks to ensure axenic culture, and confirmation of strain identity before plant inoculation. Samples not meeting criteria are rejected and disposed, and new aliquots are drawn from the Master Bank. If a problem is identified at the Master Bank level, a new Master Bank is generated and subjected to quality control procedures before further use.

Step 1b. Ethanol induction of transgenic plants

In this variation of the method, transgenic plants carrying an ethanol-inducible promoter are used. The procedure was developed by Notifier and described by Werner (2011). The process is based on inducible release of viral RNA replicons from stably integrated DNA pro-replicons. A simple treatment with dilute ethanol releases the replicon leading to RNA amplification and high-level production of the desired thaumatin protein.

Step 2. Host plant preparation

For agroinduction, normal seeds of *Spinacia oleracea* (spinach), *Beta vulgaris* (beet) or *Lactuca sativa* (lettuce), or other adequate production host plant are obtained from qualified seed producers. For ethanol induction, transgenic seeds of these host plants developed by Notifier are used, which contain the gene insert for the desired thaumatin driven by an ethanol-inducible promoter.

With either method of induction, plants are propagated in trays using a food-crop compatible soil based substrate, fertilizer and water. For seeding, plant propagation, target expression and plant harvest, the principles of Good Agriculture and Collection Practices (GACP) are applied. All used materials underlie a quality management system ensuring a predefined quality.

Step 3a. Inoculation of host plants with agrobacterial vector

The *A. tumefaciens* inoculum carrying the selected thaumatin replicon is applied to greenhouse-grown and quality tested host plants through the stomata (pores) in the leaves. The plant hence takes the place of a conventional “fermenter” in the production of the product. The *Agrobacterium* inoculum and the host plants are cultured under predefined and controlled conditions. At a specified timepoint after seeding, the plants are treated with a defined concentration of *Agrobacterium* in dilution buffer.

Inoculation of plants is accomplished by either vacuum-mediated infiltration after immersing the plant leaves in a suspension of the inoculum, or via a procedure wherein the inoculum is sprayed onto plant leaves mixed with a surfactant (Gleba 2014; Hahn 2015; Tusé 2014). Via either method, the agrobacteria are efficiently internalized into the plant and gain systemic distribution.

The agrobacteria infect the plant cells and insert the T-DNA plasmid into the nucleus, which initiates synthesis of thaumatin-encoding RNA transcripts. Amplification of the transcript and translation of the thaumatin RNA message into thaumatin occurs in the cytoplasm of each plant cell.

Neither the vector nor thaumatin genes are integrated into seed or passed on to subsequent generations (i.e. no stable integration); thus, the expression of proteins via viral vectors is transient and the production plants are not genetically modified (i.e., non-GMO).

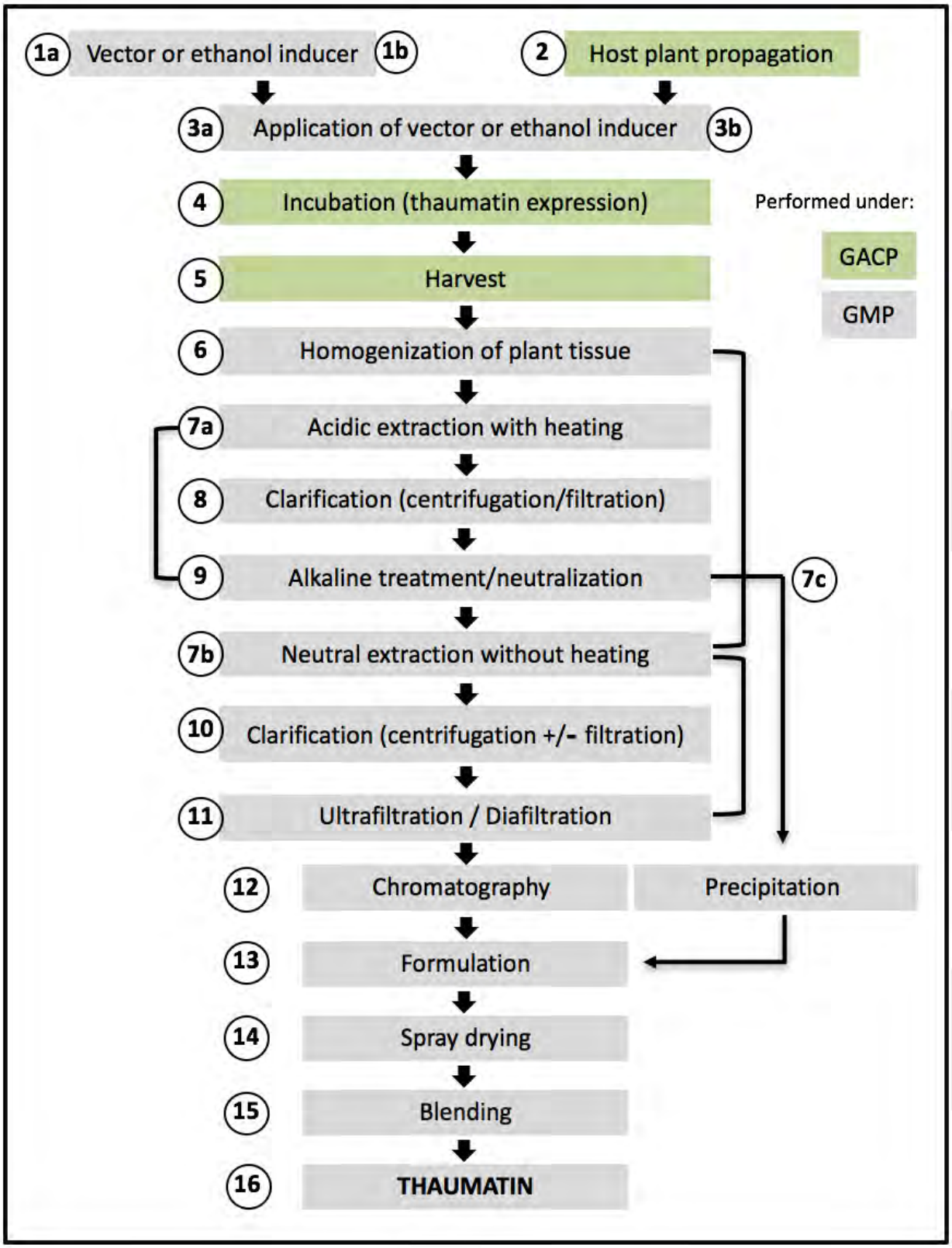

Figure B-3. Summarized process diagram for THAUMATIN production in plants

Step 3b. Ethanol induction

In this variation of the method, treatment of the transgenic plants carrying a thaumatin gene with dilute ethanol (2.5% v/v) releases the replicon leading to RNA amplification and high-level thaumatin expression. To achieve tight control of replicon activation and spread in the non-induced state, the viral vector has been deconstructed, and its two components, the replicon and the cell-to-cell movement protein, have each been placed separately under the control of an inducible promoter (Werner 2011). Throughout the induction period, thaumatin protein accumulates in the tissues of the host plant. The inducer (ethyl alcohol) is evaporated or metabolized during plant growth and is not found in the final product.

Step 4. Incubation

After agro-inoculation or ethanol induction, the plants are incubated for 5-10 days under controlled temperature, humidity and illumination to allow for accumulation of thaumatin. During this incubation, there is rapid systemic replication of the vector and expression and accumulation of the induced product.

Step 5. Harvest

Plants producing thaumatin protein are harvested typically 8-10 days post inoculation/induction. Samples of plant biomass are taken for analyses of thaumatin protein content, general health and other process QC procedures prior to large-scale extraction. Plants in trays are transported to the cutting operation. The plants' aerial biomass (i.e. leaves and part of the stems) are mechanically cut and harvested into bins, which are transported to the extraction room.

Step 6. Homogenization of plant tissue

Cut plant biomass is disintegrated by homogenization in a grinder using an extraction buffer; the course plant material and fibers are removed, and the protein-containing soluble stream is further purified through a series of physicochemical treatments, precipitations and filtration steps.

There are three (3) options for extracting thaumatin from plant biomass, described in Steps 7a-9, alternatively in Steps 7b-11, or alternatively in Steps 7c and 13.

Step 7a. Acidic extraction

The complex stream from Step 6 is subjected to low pH treatment with heating to 60 °C to help precipitate major host cell proteins, resulting in a partially purified stream enriched for the thaumatin protein. Thaumatins' heat stability under acidic conditions greatly assists the efficiency of purification by this method.

Step 8. First clarification

Precipitated proteins and other impurities are removed by centrifugation and/or filtration.

Step 9. Neutralization

After clarification in Step 8, the process stream is pH-adjusted with alkali for further processing.

Step 10. Second clarification

The solution from Step 9 is further clarified by centrifugation and/or filtration.

Step 11. Ultrafiltration / diafiltration

Additional impurities are removed by ultrafiltration and diafiltration; typically, impurities that are less than 5-10 kDa in mass are eliminated at this step.

Step 7b. Neutral extraction

An alternative isolation procedure to that described in Step 7a is to subject the homogenate from Step 6 to neutral extraction at low temperature. Precipitating proteins and other cellular debris are removed by centrifugation or filtration to enrich the thaumatin-containing supernatant/filtrate. This is followed by Step 10 and Step 11 wherein filtration (ultrafiltration or diafiltration) removes most remaining host impurities.

Step 7c. Precipitation

Yet another thaumatin isolation procedure entails precipitation from clarified plant extracts with tartrate (GRAS) by the method of Asherie et al (2008), which yields highly pure, mature crystalline thaumatins.

Step 12. Chromatography

Optionally, the product-enriched solution can be further purified by SP-Sepharose HP chromatography.

Steps 13 – 16. Formulation, drying, blending, fill and finish

The final THAUMATIN precursor solution is stabilized and standardized by the addition of water, food-compatible pH regulators, as needed. The solution is filter-sterilized and either spray dried or lyophilized to produce a dry, off-white, yellowing to light tan powder. In a mixed-thaumatin formulation, the levels of thaumatin I and II can be adjusted by blending. Prior to release, the bulk product is tested to ensure compliance with the final product specification.

In-Process controls and quality assurance

Notifier applies rigorous in-process controls to manage the quality of process intermediates and final products throughout the manufacturing process. Materials not meeting pre-determined specifications are rejected. Product release is done after each batch passes rigorous identity and purity tests. A Quality Management system is in place to ensure conformance with industry standards and federal and local regulatory guidelines.

B.4. Manufacturing Facilities

Notifier can manufacture THAUMATIN at various locations in Europe and the United States. For commercial manufacture, semi-automated plant cultivation, inoculation, incubation and harvesting systems can be applied. Depending on the scale needed, Notifier can manufacture at its own facilities or use a contract manufacturing organization to produce and formulate thaumatin proteins meeting Notifier's specification. Features of an existing US facility's upstream and downstream processing capabilities include:

Upstream

- 80,000 sq ft of controlled growth space with 672 tables holding 30,240 plant trays in 3 levels. Each tray holds 104 plants; controlled conditions for the growth and harvest of transfected plants
- An automated plant transport system allowing movement, irrigation, lighting and environmental control (temperature and humidity) of trays for plant growth

Downstream

- 32,000 sq ft manufacturing area
- Linear scalability: 1 metric ton (mt)/shift pilot scale – 68 mt/shift commercial scale
- 75 L of Green Juice (post-grind/pre-clarification extract) per minute; continuous processing up to UF
- 35,000 L of tank storage capacity; heating and cooling control of in-process material
- Manufacturing clean rooms with controlled environments
- Computer-controlled processing and data collection
- Clarification options (UF/DF/Microfiltration/Nanofiltration/Reverse Osmosis)

Regardless of manufacturing venue, all substances, materials and reagents used in manufacturing THAUMATIN by Notifier's process conform to food grade or higher standards. All processing equipment is high-grade stainless steel meeting food-industry criteria. All cleaning and sterilization procedures are validated in accordance with FDA guidelines for food-grade materials.

B.5. Waste Handling and Disposal

Waste streams containing plant-derived residuals are treated per local regulations and discarded. No by-products of the process are used in food or feed products, supplements, additives or processing aids.

B.6. Specification

A Specification for the plant-produced THAUMATIN product (final bulk) produced through the manufacturing process described herein is shown in Table B-1. THAUMATIN is provided in bulk as a dry powder and consists of thaumatin I and/or II. On a molar basis, the taste-modifying effects of thaumatins I and II are interchangeable; THAUMATIN bulk can consist of either protein or a mixture thereof.

Table B-1. Specification for THAUMATIN Product

THAUMATIN Bulk Product		
Parameter	**Specification limit**	**Method**
Appearance	Powder, yellowish to light tan	Visual
Minimum total thaumatin content (percent of total protein)	≥98%	HPLC
Solubility (DI water)	600 mg/mL	Visual
pH of a 1% solution (excipient-dependent)	2.7 – 6.0	Potentiometric
Heavy metals (sum of Ag, As, Bi, Cd, Cu, Hg, Mo, Pb, Sb, Sn)	≤30 ppm	USP38<233>
Lead	≤5 ppm	USP38<233>
Bioburden	≤5,000 CFU total per g	USP32<61>
Agrobacterium per 10 g sample	0 (absent)	Selective plate-based assay
Undesirable microorganisms, including *Escherichia coli, Pseudomonas aeruginosa, Salmonella spp.* or coagulase-positive *Staphylococcus spp.*, per 25 g	0 (absent)	USP32<1111>
Stability (dry concentrate; 0-10°C)	>6 months	HPLC; thaumatin peak in 1% solution at T_n relative to T_0

APPENDIX C. THAUMATIN Characterization

The thaumatin proteins expressed in the process described in APPENDIX B were analyzed for quality independently and for equivalency to *Thaumatococcus*-derived thaumatins, both extracted and recombinant, using publicly available information on thaumatin sequence and structure. Representative characterization methods and results are included. Although Notifier's thaumatin I and thaumatin II have been characterized by these methods, for brevity some of the results are shown only for thaumatin II; the results with thaumatin I are analogous.

Purification from plant biomass

The plant homogenates obtained as described in APPENDIX B are further purified through a series of physicochemical treatments, precipitations, crystallizations and/or filtration steps. Three methods were evaluated for extracting thaumatin with respect process efficiency and the quality of the extracted product.

Method 1: Acidic extraction with heat. The biomass slurry is subjected to low pH treatment (pH 4) in aqueous buffer with heating to 60 °C for 10-60 min to precipitate major host cell proteins, resulting in a process stream that is enriched for the thaumatin protein. Thaumatins' heat stability under acidic conditions greatly assists the efficiency of purification by this method.

Figure C-1 shows representative results for the purity of thaumatin II achieved by heat extraction, as assessed in 15% SDS gel. Expression yield for these studies was 1.5 g thaumatin per kg plant biomass wet weight. This represents 30-50% of the plants total soluble protein (TSP). In an informal tasting, the taste of the isolated thaumatin was comparable to that of commercial reference material.

Figure C-2 shows purification of thaumatin II by heat extraction in water or PBS. Purity of mature thaumatin II (THM2; labelled 3) is shown in relation to THM2 pre-proprotein (1) and proprotein (10).

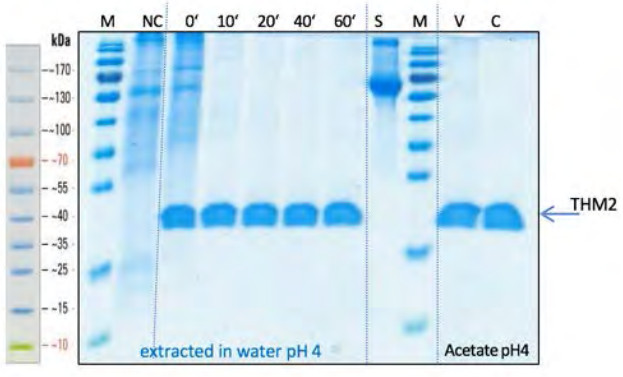

Figure C-1. Expression yield of thaumatin II (THM2) achieved with one-step heat extraction

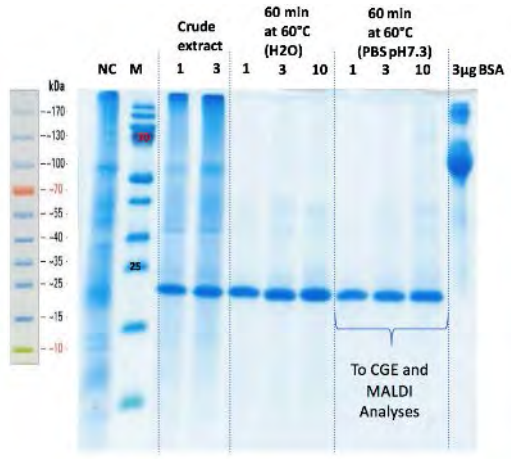

Figure C-2. Purity of mature thaumatin II (THM2) obtained by heat extraction

Samples of heat-purified THM2 were subjected to further purification by **capillary gel electrophoresis (CGE)** and subsequently isolated protein was subjected to MALDI and tryptic-MALDI analysis. Figure C-3 shows results of CGE analysis.

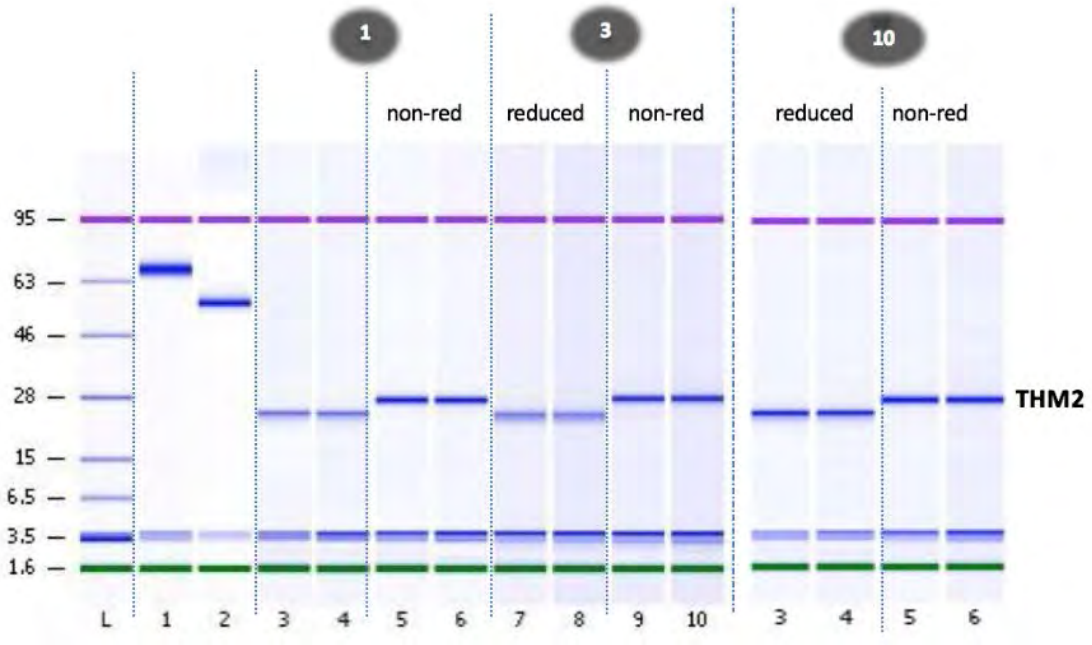

Figure C-3. Purity of heat-extracted thaumatin II by CGE

BSA was used as a standard. Sample 1 contains thaumatin II (THM2) pre-proprotein with native signal peptide (SP); Sample 3 contains THM2 mature protein with rice ATS, and Sample 10 contains THM2 proprotein with rice SP. No other protein bands appear in thaumatin samples; THM2 is >98% purity.

Figure C-4 shows CGE chromatograms of mature thaumatin II (non-reduced, left; reduced, right), illustrating the high purity of the protein and that the mature protein is of the expected molecular mass.

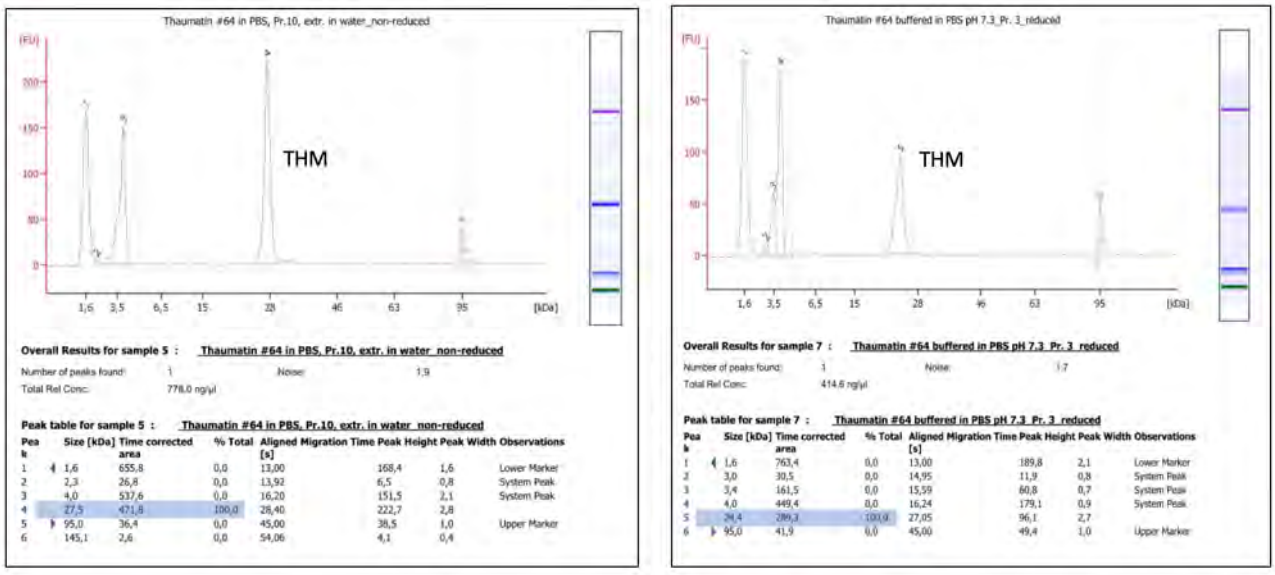

Figure C-4. CGE Chromatograms of thaumatin II

CGE chromatograms of 100% mature thaumatin II protein under non-reducing (left panel, Peak 4) and reducing (right panel, Peak 5) conditions. Green and magenta colored bands indicate lower and upper MW markers. No other protein bands appear in the thaumatin sample, underscoring the efficiency of the 60 °C water extraction method.

Confirmation of identity

The heat-process isolated thaumatin was subjected to **MALDI-TOF** and **tryptic MALDI MS** analyses to determine the molecular mass of the expressed protein, and to confirm that accessory sequences and signal peptide were cleaved to yield the expected mature protein.

Figure C-5 shows representative results. There are slight discrepancies among the molecular masses estimated using CGE and mass spectrometry, with MS yielding the more precise values. Mass values also depend on the conformation of the protein; for example, formation of disulfide bonds leads to loss of protons and hence lower masses. Regardless, the average molecular masses determined by various methods match the values in reference databases.

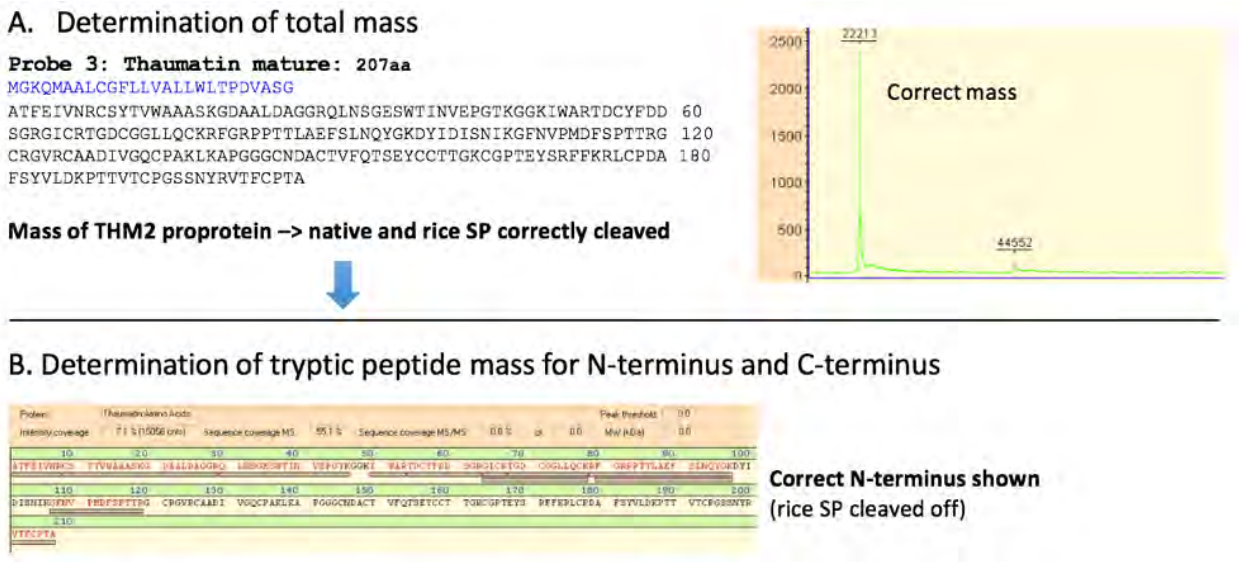

Figure C-5. Determination of thaumatin mass by MALDI-TOF

Top panel (A) shows results of MALDI-TOF analysis for total mass of the protein. Expression of the pre- and preproprotein leads to post-translational processing *in planta* to yield the expected mature thaumatin. The signal peptide added to aid expression and target accumulation is cleaved off to yield a product with the expected mass. Bottom panel (B) shows typical results of N- and C-terminal tryptic mass fingerprinting, confirming total mass and the cleavage of accessory sequences to yield the expected mature protein.

Method 2: Cold extraction at neutral pH. After clarification of the process stream by centrifugation and/or filtration, the semipurified thaumatin-containing solution is subjected to SP Sepharose HP chromatography, to yield a pure and homogeneous product.

The chromatographic separation method takes advantage of thaumatins' high isoelectric points (pI 11.5-12.5). A strong cation exchange (CEX) resin such as SP Sepharose HP (GE Healthcare) is used and results in a highly pure and homogeneous protein that can be readily and economically recovered. Figure C-6 shows typical results of the one-step chromatographic purification.

Thaumatins isolated by the chromatographic method show the same mass, amino acid sequence and purity as thaumatins isolated by the heat-precipitation method. Thaumatins are highly stable to heat at acidic pH, and are stable during chromatographic separation at low temperature. Either isolation method yields protein with consistent and defined characteristics.

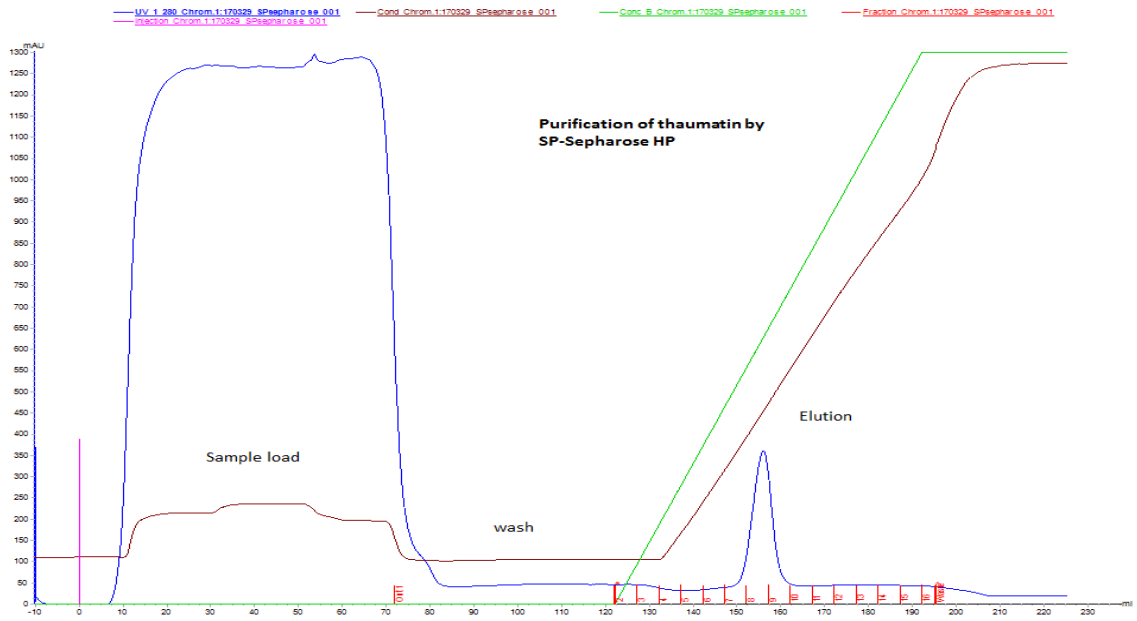

Figure C-6. One-step purification of cold-extracted thaumatin by SP Sepharose HP chromatography

Chromatographic purification of thaumatin I or II (pI 11.5-12.5) with the strong cation exchange resin in SP Sepharose HP (GE Healthcare) results is highly pure and homogeneous protein

In addition to CGE, a more precise method of identity confirmation of plant-produced thaumatins, *in situ* protein digestion (ISD), was also performed to assess thaumatin integrity after purification. Representative results are shown in Figure C-7, which confirm the stability of the 8 disulfide bridges and the lack of truncation.

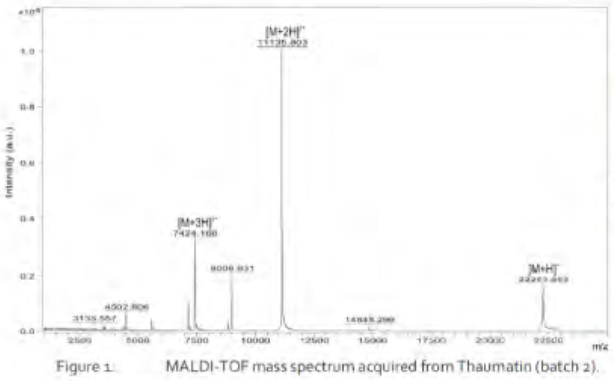

Figure 1: MALDI-TOF mass spectrum acquired from Thaumatin (batch 2).

Table 1 – Mass signals for Thaumatin (batch 2)

	$[M+H]^+$	$[M+2H]^{2+}$	$[M+3H]^{3+}$	$[M+4H]^{4+}$	Mean
m/z	22263.9	11135.8	7424.2	5568.4	-
Mass (Da)	22262.9	22269.6	22269.5	22269.7	**22267.9**
Dev. (Da)	-9.0	-2.3	-2.4	-2.2	**-4.0***
Modification	• Cleavage of N-terminal signal sequence				

*Deviation = $M_{Avg.,\ experimental} - M_{Avg.,\ theoretical}$ (22271.9 Da)

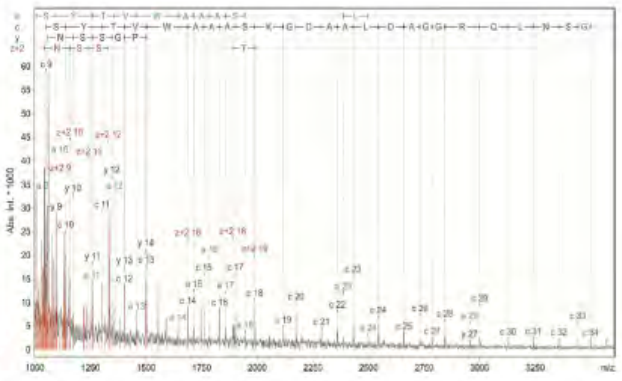

Figure 2: Section of the ISD fragment spectrum acquired from Thaumatin (batch 2).

- Only 1 proteoform present when apoplast targeting sequence is cleaved
- 8 disulfide bonds intact
- No N- and C-terminal truncation

Figure C-7. Stability of thaumatin after purification as determined by ISD

Method 3: Precipitation of thaumatin with tartrate. This method is an adaptation of the method described by Asherie et al (2008), in which tartrate (a GRAS substance) is used to precipitate thaumatin from solution. Figure C-8 shows typical structures of thaumatin crystalline precipitates obtained by tartrate addition. Crystals represent pure protein.

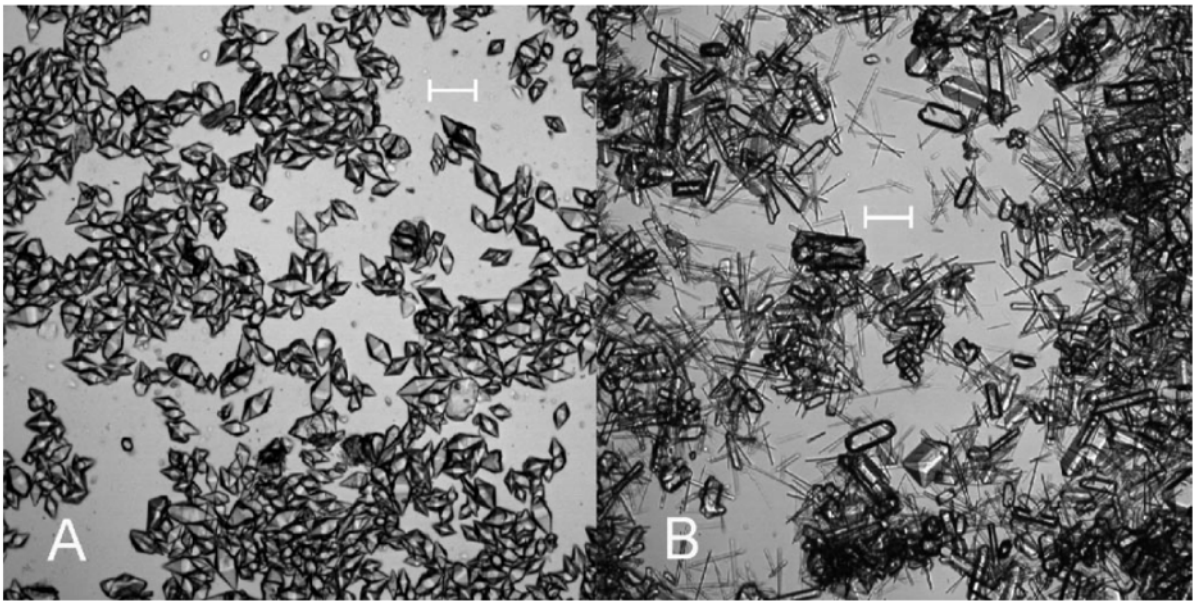

Figure C-8. Crystals of thaumatin obtained by precipitation with tartrate

Thaumatin can be isolated from clarified process stream by precipitation induced by addition of tartrate salts under controlled pH, temperature and incubation conditions. The images show crystals of thaumatin produced by enantiomerically pure tartrate precipitants. Left panel (A) shows bipyramidal crystals formed in 0.5 M sodium L-tartrate, while right panel (B) shows prismatic and stubby crystals formed in 0.5 M sodium D-tartrate (detailed description of the method is found in Asherie 2008). Scale bars for the two images represent 50 µm.

In Notifier's adaptation, the clarified process stream from either *Agrobacterium*- or ethanol-induced plants (Step 7c in Figure B-3), are treated with tartrate under temperature and pH controlled conditions to induce precipitation of thaumatin crystals, which can then be recovered by centrifugation or filtration, and further formulated to meet the specification of the THAUMATIN product. Analytical studies to characterize thaumatin proteins recovered by cold precipitation were in progress at the time of this submission.

Summary

In summary, the sweet/flavor-modifying thaumatin proteins that comprise Notifier's THAUMATIN product are expressed with signal peptide to enhance their accumulation in plant tissue and facilitate isolation. Each precursor protein is correctly processed *in planta* post-translationally to produce the final mature protein.

Any of the three methods used for purifying thaumatins from plant biomass (heat/acid-induced precipitation, cold chromatography, or tartrate-induced crystallization) yields highly pure product (>98%).

The amino acid composition, amino acid sequence, and molecular mass of the resultant proteins are identical to the corresponding thaumatins I and II found in *Thaumatococcus daniellii*, which form the basis of currently marketed thaumatin products.

The methods used to characterize thaumatins, summarized in this section of the Notice, are industry standard and are validated and conducted by licensed external analytical laboratories. Other release tests are applied to each batch of thaumatin (not shown here) to ensure that the product meets its specification. These analyses include total thaumatin protein content, pH, heavy metal content, and bioburden.

It is important to note that thaumatin products approved as food additives in multiple countries are not 100% pure thaumatin, and contain a number of host- and process-derived impurities (Lim 2012). Regardless, the safety of the commercial products has been conclusively shown and, by comparison, the safety of Notifier's final product has also been affirmed (see Section 6.1 (overall safety), Section 6.2 (allergenic potential), and Section 6.3 (safety in relation to dietary intake)).

Aqueous extracts of the seed aril of earlier commercial products containing *Thaumatococcus* thaumatins contained, in addition thaumatin proteins, a cysteine protease designated thaumatopain (Cusack 1991). Thaumatopain was found to be a monomeric protein of MW 30 kDa. The protease strongly resembled papain in proteolytic activity, pH optima, susceptibility to inhibitors of cysteine proteases and in N-terminal sequence. Thaumatopain was found responsible for the cysteine protease activity previously attributed to thaumatin. Thaumatin was digested by thaumatopain at neutral to alkaline pH values. Thaumatin has no intrinsic proteolytic activity; the proteolytic activity in partially purified thaumatin preparations was attributable to thaumatopain, that needs to be separated from thaumatin by chromatography (Stephen 1991).

Thaumatococcus daniellii aril gel is partially solubilized during extraction and may contaminate the final product, albeit at low levels. The gel was found to contain residues of L-arabinose, D-xylose, D-glucuronic acid, and 4-O-methyl-D-glucuronic acid, together with nitrogen (1%) and ash (3.1%) (Adesina 1977). The ash-free gel contained 76% pentose and 24% uronic acid; 25% of the uronic acid occurred as the 4-O-methyl derivative. The leaf, flower and fruit coat of *T. daniellii* contain derivatives of flavonol and flavone (Adesina 1978). These compounds are absent in the rhizome and fruit parts (e.g. aril, gel and seed).

Importantly, impurities found in some commercial *Thaumatococcus* extracts are not found in the species used by Notifier for manufacturing THAUMATIN. Further, Notifier's recombinant process is inherently different from extraction of a wild species, and process-derived impurities or contaminants reported for *T. daniellii*-derived thaumatins would not be found in Notifier's end product. Trace levels of plant sugars or proteins that might be present are at such low levels that they are not expected to pose a safety risk, especially at the projected low (ppm; mg/kg) application rates of the THAUMATIN product.

Conclusion

Taken together, results of internal characterization tests of the thaumatin proteins produced by the methods described in this Notice show that the sweetening and flavor-modifying components in Notifier's THAUMATIN product, thaumatin I and/or thaumatin II, are identical in structure and composition to those found in commercially approved thaumatin preparations.

The results of analyses conducted by Notifier allow us to compare the characteristics of our product to the characteristics of extracted and recombinant thaumatins as found in the extensive public record for these proteins. We therefore conclude that thaumatins as found in Notifier's THAUMATIN product are GRAS when produced through the methods described and used at the recommended application rates allowed in foods and beverages in the USA and multiple other countries.