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SENT *VIA* FEDEX

September 20, 2011

Robert L. Martin, Ph.D.
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food And Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Re: Re-Submission of GRAS Notice for 1,4-α-Glucan Branching Enzyme Preparation from *Bacillus subtilis* Expressing the 1,4-α-Glucan Branching Enzyme Gene from *Aquifex aeolicus* (Original GRN 000361)

Dear Dr. Martin:

In accordance with proposed 21 CFR §170.36 [Notice of a claim for exemption based on a Generally Recognized As Safe (GRAS) determination] published in the *Federal Register* [62 FR 18938 (17 April 1997)], I am submitting in triplicate, as the notifier (Ezaki Glico Co., Ltd., 4-6-5, Utajima, Nishioyogogawa, Osaka, Japan], a Notice of the determination, on the basis of scientific procedures, that Ezaki Glico's 1,4-α-glucan branching enzyme preparation from *Bacillus subtilis* expressing the 1,4-α-glucan branching enzyme gene from *Aquifex aeolicus*, as defined in the enclosed documents, is GRAS under specific conditions of use as a processing aid for use in the production of food ingredients (*e.g.,* cyclic dextrin, highly branched), and therefore, is exempt from the premarket approval requirements of the *Federal, Food, Drug and Cosmetic Act.* The enclosed Notice replaces the original Notice for 1,4-α-glucan branching enzyme, which was submitted on October 7, 2010 and subsequently withdrawn on January 31, 2011 (GRN 000361). Information setting forth the basis for the GRAS determination, which includes a comprehensive summary of the data available and reviewed by an independent panel of experts in support of the safety of 1,4-α-glucan branching enzyme preparation from *Bacillus subtilis* expressing the 1,4-α-glucan branching enzyme gene from *Aquifex aeolicus* under the intended conditions of use, also are enclosed.

I would also like to bring to your attention that Dr. Paul Kuznesof was an Expert Panel member that undertook the GRAS determination of cyclic dextrin, highly branched, incorporating the use of 1,4-α-glucan branching enzyme preparation from *Bacillus subtilis* expressing the 1,4-α-glucan branching enzyme gene from *Aquifex aeolicus*, on behalf of Ezaki Glico. While Dr. Paul Kuznesof was in a position to sign the Expert Opinion Statement for the cyclic dextrin, highly branched GRAS notification, which is being filed currently, unfortunately, due to his untimely passing, was unable to sign the enclosed Expert Opinion Statement for 1,4-α-glucan branching enzyme preparation from *Bacillus subtilis* expressing the 1,4-α-glucan branching enzyme gene from *Aquifex aeolicus*.

I trust that the enclosed Notice is acceptable. Should you have any questions or concerns regarding this GRAS Notice, please do not hesitate to contact me at any point during the review process so that we may provide a response in a timely manner. FDA correspondence

Sincerely,
(b) (6)

Takashi Kuriki
Chief Scientific Officer

Encl.

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Generally Recognized as Safe (GRAS) Exemption Claim for 1,4-α-Glucan Branching Enzyme Preparation from *Bacillus subtilis* Expressing the 1,4-α-Glucan Branching Enzyme Gene from *Aquifex aeolicus* for Use as a Processing Aid in Food Production

Submitted to: Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition (CFSAN)
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD
U.S.A. 20740-3835

Submitted by: Ezaki Glico Co., Ltd
4-6-5, Utajima, Nishioyogogawa
Osaka, Japan

September 20, 2011

Generally Recognized as Safe (GRAS) Exemption Claim for 1,4-α-Glucan Branching Enzyme Preparation from *Bacillus subtilis* Expressing the 1,4-α-Glucan Branching Enzyme Gene from *Aquifex aeolicus* for Use as a Processing Aid in Food Production

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I GRAS EXEMPTION CLAIM

I.A Claim of Exemption from the Requirement for Premarket Approval Pursuant to Proposed 21 CFR §170.36(c)(1) [62 FR 18938 (17 April 1997) (U.S. FDA, 1997)]

1,4-α-Glucan branching enzyme preparation [1,4-α-D-glucan: 1,4-α-D-glucan 6-α-D-(1,4-α-D-glucano)-transferase; EC 2.4.1.18; CAS No. 9001-97-2] from *Bacillus subtilis* expressing the 1,4-α-Glucan branching enzyme gene from *Aquifex aeolicus* has been determined to be Generally Recognized as Safe (GRAS) by Ezaki Glico Co., Ltd. (Ezaki hereafter) for use as a processing aid in the production of food ingredients, consistent with Section 201(s) of the *Federal Food, Drug, and Cosmetic Act.* This determination is based on scientific procedures as described in the following sections. Therefore, the use of the 1,4-α-glucan branching enzyme preparation in food production as described below is exempt from the requirement of premarket approval.

Signed,

(b) (6)

September 22, 2011
Date

Takashi Kuriki
Chief Scientific Officer
Ezaki Glico Co., Ltd
4-6-5, Utajima, Nishioyogogawa
Osaka, Japan

I.B Name and Address of Notifier

Takashi Kuriki
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I.C Common Name of the Notified Substance

The common name of the notified substance is 1,4-α-glucan branching enzyme preparation.

I.D Conditions of Intended Use in Food

The 1,4-α-glucan branching enzyme preparation from genetically-modified *B. subtilis* 168 expressing the 1,4-α-glucan branching enzyme gene from *A. aeolicus* VF5 is intended for use as a processing aid in food production. Specifically, the 1,4-α-glucan branching enzyme preparation is intended for use in the production of modified food starch ingredients, including highly-branched cyclic dextrins (also known as cyclic dextrin, highly branched[1]) produced from food-grade waxy corn starch, at a typical use level of 500 U/g substrate. The branching enzyme preparation is used only at levels necessary to achieve the intended effect.

I.E Basis for the GRAS Determination

Pursuant to Title 21, Section 170.30 of the *Code of Federal Regulations* (CFR) (U.S. FDA, 2011), 1,4-α-glucan branching enzyme preparation from genetically-modified *B. subtilis* 168 expressing the 1,4-α-glucan branching enzyme gene from *A. aeolicus* VF5 has been determined to be GRAS based on scientific procedures for use in the production of cyclic dextrin, highly branched.

This GRAS determination is based on data generally available in the public domain pertaining to the safety of the branching enzyme, as discussed herein, and on consensus among a panel of experts who are qualified by scientific training and experience to evaluate the safety of the 1,4-α-glucan branching enzyme preparation for use as a processing aid in food production [see Appendix A, entitled "Expert Panel Consensus Report Regarding the Generally Recognized as Safe (GRAS) Status of 1,4-α-D-Glucan Branching Enzyme Preparations Derived from *Bacillus stearothermophilus* or Genetically-Modified *B. subtilis* with the Branching Enzyme Gene from *B. stearothermophilus or Aquifex aeolicus* for Use as a Processing Aid in the Manufacture of Cyclic Dextrin, Highly-Branched and Enzymatically-Synthesized Glycogen"].

At the request of Ezaki, an Expert Panel ("the Expert Panel") of independent scientists, qualified by their relevant national and international experience and scientific training to evaluate the safety of food ingredients, was specially convened to conduct a critical and comprehensive evaluation of the available pertinent data and information, and to determine whether the intended uses of the branching enzyme as a food processing aid is safe and suitable and would be GRAS based on scientific procedures.

---

[1] A GRAS Exemption Claim for cyclic dextrin, highly branched [trade name Cluster Dextrin® (CCD®)] has been concurrently submitted to FDA.

The Expert Panel consisted of the following qualified scientific experts: Dr. Joseph F. Borzelleca (Virginia Commonwealth University School of Medicine), Dr. William J. Waddell (University of Louisville School of Medicine), and Dr. Paul Kuznesof[2] (independent consultant).

The Expert Panel convened on behalf of Ezaki independently and collectively, and critically evaluated the data and information summarized herein and concluded that the intended uses in food processing described herein for 1,4-α-glucan branching enzyme preparation from genetically-modified *B. subtilis* 168 expressing the 1,4-α-glucan branching enzyme gene from *A. aeolicus* VF5, meeting appropriate food-grade specifications and manufactured according to current Good Manufacturing Practice (cGMP), are safe and suitable and GRAS based on scientific procedures. It also is the Expert Panel's opinion that other qualified and competent scientists reviewing the same publicly available toxicological and safety information would reach the same conclusion.

1,4-α-Glucan branching enzyme preparation from genetically-modified *B. subtilis* 168 expressing the 1,4-α-glucan branching enzyme gene from *A. aeolicus* VF5 is GRAS based on scientific procedures for its intended use as a processing aid in the production of the modified food starch ingredient, cyclic dextrin, highly branched; therefore, it is excluded from the definition of a food additive, and thus, may be marketed and sold for its intended purpose in the U.S. without the promulgation of a food additive regulation under Title 21 of the CFR.

I.F Availability of Information

The data and information that serve as the basis for this GRAS Notification will be sent to the U.S. Food and Drug Administration (FDA) upon request, or will be available for review and copying at reasonable times at the offices of:

Ezaki Glico Co., Ltd
4-6-5, Utajima, Nishioyogogawa
Osaka, Japan

Should FDA have any questions or additional information requests regarding this notification, Ezaki will supply these data and information.

[2] Dr. Paul Kuznesof had passed before the signing of the Expert Panel Consensus Report and preparation and submission of this GRAS exemption claim. Dr. Paul Kuznesof participated in the Expert Panel meeting held for the cyclic dextrin, highly branched ingredient at which the GRAS status of the 1,4-α-glucan branching enzyme preparation was discussed and concluded. Please see the concurrently submitted GRAS Exemption Claim for cyclic dextrin, highly branched [trade name Cluster Dextrin® (CCD®)] and its accompanying Expert Panel Consensus Report, as well as the letter provided in Appendix A.

II. DETAILED INFORMATION ABOUT THE SOURCE AND IDENTITY OF THE SUBSTANCE

II.A Identity

II.A.1 Identity of the Enzyme Preparation

The 1,4-α-glucan branching enzyme preparation is produced using *B. subtilis* 168 genetically modified to express the 1,4-α-glucan branching enzyme structural gene from *Aquifex aeolicus* VF5. The gene that encodes the 1,4-α-glucan branching enzyme is *glgB*. The branching enzyme preparation is a dark-brown solution, and is identified as follows:

Common or Usual Name:	1,4-α-Glucan branching enzyme
Trade Name:	Not applicable
Chemical Name:	1,4-α-D-Glucan: 1,4-α-D-glucan 6-α-D-(1,4-α-D-glucano) transferase
International Union of Biochemistry and Molecular Biology (IUBMB) Enzyme Nomenclature:	1,4-α-D-Glucan branching enzyme
IUBMB Number: [Enzyme Commission (EC) Number]	2.4.1.18
Chemical Abstracts Service (CAS) Number:	9001-97-2

The 1,4-α-glucan branching enzyme preparation is produced using standard genetic modification techniques as described in Section II.B, but is not modified by protein engineering, post-translational processes, or other technological processes. 1,4-α-Glucan branching enzyme has a molecular weight between 62,000 and 66,000 g/mol and its amino acid sequence is as follows:

```
MKKFSLISDYDVYLFKEGTHTRLYDKLGSHVIELNGKRYTFFAVWAPHADYVSLIGDFNEWDKGSTPMVK
REDGSGIWEVLLEGDLTGSKYKYFIKNGNYEVDKSDPFAFFCEQPPGNASVVWKLNYRWNDSEYMKKRKR
VNSHDSPISIYEVHVGSWRRVPEEGNRFLSYRELAEYLPYYVKEMGFTHVEFLPVMEHPFYGSWGYQITG
YFAPTSRYGTPQDFMYLIDKLHQEGIGVILDWVPSHFPTDAHGLAYFDGTHLYEYEDWRKRWHPDWNSFV
FDYGKPEVRSFLLSSAHFWLDKYHADGLRVDAVASMLYLDYSRKEWVPNIYGGKENLEAIEFLRKFNESV
YRNFPDVQTIAEESTAWPMVSRPTYVGGLGFGMKWNMGWMNDTLFYFSKDPIYRKYHHEVLTFSIWYAFS
ENFVLPLSHDEVVHGKGSLIGKMPGDYWQKFANLRALFGYMWAHPGKKLLFMGGEFGQFKEWDHETSLDW
HLLEYPSHRGIQRLVKDLNEVYRREKALHETDFSPEGFEWVDFHDWEKSVISFLRKDKSGKEIILVVCNF
TPVPRYDYRVGVPKGGYWREIMNTDAKEYWGSGMGNLGGKEADKIPWHGRKFSLSLTLPPLSVIYLKHEG
```

II.A.2 Properties of the Enzyme Preparation

Branching enzymes occur naturally in plants, microorganisms, and animals, as well as in humans. For instance, 1, 4-α-glucan branching enzyme present in plants is utilized for the formation of the amylopectin component of starch (Borovsky *et al.*, 1975, 1976), while in humans (Thon *et al.,* 1993), animals (Huijing, 1975), and microorganisms (Preiss, 1984), the branching enzyme is involved in glycogen formation.

The 1,4-α-glucan branching enzyme preparation, produced using genetically-modified *B. subtilis* 168 expressing the branching enzyme gene from *A. aeolicus* VF5, is thermostable, with optimal activity at a temperature of 75°C and pH ranging from 7.5 to 8.0 (Takata *et al.*, 2003). 1,4-α-Glucan branching enzyme is responsible for catalyzing an intra- or intermolecular transglycosylation reaction in glucan substrates, resulting in the formation of an α-(1,6) glucosidic bond. The substrate for the enzyme is α-amylase-hydrolyzed food-grade waxy corn starch, which is predominantly in the form of amylopectin, with an amylose content of less than 1% (Buléon *et al.*, 1988). Isoamylase-hydrolyzed food-grade dextrin (derived from corn starch) may also be used as a substrate.

The enzyme activity of 1,4-α-glucan branching enzyme preparation is 40,000 U/mL±10,000, and is highly specific to branching enzyme activity. The catalytic residues of 1,4-α-glucan branching enzyme consist of 2 aspartic acid (Asp) residues and 1 glutamic acid (Glu) residue. These residues are conserved in bacterial species, with specific positioning at Asp-311, Asp-430, and Glu-362 in the 1,4-α-glucan branching enzyme expressed by *A. aeolicus* VF5. Analytical data supporting the activity level of the enzyme is provided in Table II.A.3.3-1, and demonstrate that the material produced by the manufacturing process is consistent and complies with the pertinent product specifications. The enzyme assay method is based on the ability of the enzyme to decrease the absorbance of amylose-iodine complexes, using amylose as the reference substrate. A detailed description of the assay method is provided in Appendix B.

II.A.3 Characterization of the Enzyme Source

II.A.3.1 Details on the Production Microorganism

The production strain is *B. subtilis* strain 168 genetically modified to express the gene encoding the 1,4-α-glucan branching enzyme (*glgB*) of *A. aeolicus* using standard genetic modification techniques. The production strain is non-pathogenic and non-toxicogenic, and meets the safety criteria developed by Pariza and Foster (1983), Pariza and Johnson (2001), and the International Food Biotechnology Council (IFBC) (1990) as discussed later in Section II.A.3. Details on the construction of the production organism, including the recipient and donor microorganisms, the newly introduced DNA, the genetic modification process, information on the vector used, and on the construction of the vector expression system, are described in the sections that follow.

II.A.3.1.1 Recipient Strain

The recipient, or host, strain is *B. subtilis* strain 168. *B. subtilis* 168 was obtained from the National Collection of Industrial, Food and Marine Bacteria (NCIMB; accession number 12378) and also is deposited in the American Type Culture Collection (ATCC; accession number 33677). The current taxonomic classification of *B. subtilis* 168 is presented in Table II.A.3.1.1-1.

Table II.A.3.1.1-1 Taxonomic Classification of *Bacillus subtilis* 168

Taxonomy	Taxonomic Assignment
Kingdom	Bacteria
Phylum	Firmicutes
Class	Bacilli
Order	Bacillales
Family	Bacillaceae
Genus	*Bacillus*
Species group	*Bacillus subtilis* group
Species	*Bacillus subtilis*
Subspecies	*Bacillus subtilis* subsp. *subtilis*
Strain	*Bacillus subtilis* subsp. *subtilis* str. 168

II.A.3.1.2 Donor Strain

The *glgB* gene, encoding for the 1,4-α-glucan branching enzyme, was chemically synthesized based on the amino acid sequence of 1,4-α-glucan branching enzyme as expressed by *A. aeolicus* strain VF5. The current taxonomic classification of *A. aeolicus* VF5 is presented in Table II.A.3.1.2-1.

Table II.A.3.1.2-1 Taxonomic Classification of *Aquifex aeolicus* VF5

Taxonomy	Taxonomic Assignment
Kingdom	Bacteria
Phylum	Aquificae
Class	Aquificae (class)
Order	Aquificales
Family	Aquificaceae
Genus	*Aquifex*
Species	*Aquifex aeolicus*
Strain	*Aquifex aeolicus* str. VF5

The donor strain, *A. aeolicus* VF5, was not obtained for the purposes of cloning the 1,4-α-glucan branching enzyme *glgB* gene. Instead, the *glgB* gene was chemically synthesized based on the amino acid sequence of 1,4-α-glucan branching enzyme from *A. aeolicus* VF5 as previously described by Deckert *et al.* (1998). The *glgB* gene was synthesized with a *Bam*HI

site (underlined) and a ribosome binding sequence (Shine Dalgarno sequence; doubly underlined) inserted upstream of the transcriptional start site (italicized) and an *Eco*RI site (underlined) inserted downstream of the stop codon (italicized) as follows:

> CTTGGATCCTGATTAAAAGGAGGTAATAACAT*ATG*...(coding sequences)...*TAA*TGAATTCTTC

The full sequence of the DNA insert is provided in Appendix C. Modifications to the nucleotide sequence of the *glgB* gene of *A. aeolicus* VF5 were performed as highlighted in Appendix C. These modifications were performed in order to optimize expression of the gene in *B. subtilis*.

II.A.3.2 Construction of the Vector Expression System (pUAQ2)

The vector expression system (*i.e.,* pUAQ2) was constructed using the well-characterized vector pUB110. The 1,929 base pair synthetic DNA insert was digested with *Bam*HI and *Eco*RI restriction endonucleases, and then inserted between the *Bam*HI and *Eco*RI restriction sites of vector pUB110, using T4 DNA ligase to construct the plasmid pUAQ2. One copy of the DNA insert was incorporated into the vector expression system. Expression of the 1,4-α-glucan branching enzyme was placed under the regulation of a promoter sequence contained within the pUB110 vector; therefore, the inserted sequence was limited to that required to perform the intended function (*i.e.*, to express the 1,4-α-glucan branching enzyme), and no other DNA sequence was incorporated. The full sequence of vector pUB110, as well as further details on the construction of the vector expression system, is provided in Appendix C.

II.A.3.3 Construction of the Production Microorganism

The vector expression system, pUAQ2 plasmid, is transfected into competent *B. subtilis* 168 cells according to a previously described method (Anagnostopoulos and Spizizen, 1961), without integration into genomic DNA. The 1,4-α-glucan branching enzyme producing strain was then isolated by first obtaining kanamycin-resistant colonies followed by transformation. In order to confirm successful transformation, the plasmid was extracted from selected colonies, isolated, and analyzed using restriction enzyme analysis to confirm its structure. A single successfully transformed colony was then selected in which production of 1,4-α-glucan branching enzyme production was confirmed. This clone served as the master stock for the production strain.

Vector pUB110 is a well-characterized vector that is documented to be safe for introducing new genetic material into safe recipient microbial strains, including *B. subtilis*, for the purpose of producing food enzymes (IFBC, 1990; Olempska-Beer *et al.*, 2006). Although pUB110 harbors an antibiotic (kanamycin) resistance gene, the enzyme products themselves are free of transferable antibiotic resistance gene DNA, since such resistance genes can be considered to be stably associated with the vector (IFBC, 1990), and the plasmid itself does not enter the final

food product. Moreover, the final enzyme preparation is tested for the presence of the production strain containing the kanamycin resistance gene as indicated in Section II.A.3. The presence of the antibiotic resistance gene also is of no further safety concern since the production strain does not enter the final food product and is not deliberately released into the environment; therefore, transfer of the antibiotic resistance gene to other organisms would not occur. Collectively, it can be concluded that the pUB110 vector used to carry the *glgB* from *A. aeolicus* is safe for constructing production strains that are being used in the manufacture of food enzymes.

II.A.3.4 Genetic Stability of the Production Microorganism

The genomic structure of *B. subtilis* 168 is documented to be very stable under normal growth conditions (Itaya, 1993). Additionally, repeated transformation of *B. subtilis* 168 is reported to have no effect on the genomic stability of this strain (Itaya and Tanaka, 1991). Introduction of the pUAQ2 plasmid, constructed using vector pUB110, is not expected to result in genetic instability of the production strain, considering that vector pUB110 has been established to be safe for introducing new genetic material into *B. subtilis* for the purpose of producing food enzymes (IFBC, 1990; Olempska-Beer *et al.*, 2006).

II.A.3.5 Stability of the Production Strain During Storage

The master stock for the production strain is stored frozen in glycerol at -80°C at Ezaki Glico Co., Ltd. The master stock is tested at least once a year. A sample of the master stock (diluted with sterilized saline) is streaked on agar plates, colonies grown overnight at 37°C, and analyzed for colony morphology (*i.e.,* colonies with irregular shapes or colors). Plasmids are then isolated from selected colonies and tested for resistance to kanamycin for confirmation of the production strain using the method described in Appendix B. Stability of the plasmid structure is also analyzed with the use of restriction endonucleases to confirm that no modifications occurred during storage. Selected colonies are also tested for acceptable 1,4-α-glucan branching enzyme productivity.

II.A.4 Composition of the Enzyme Preparation

II.A.4.1 Levels of Diluents, Stabilizers, and Preservatives

The 1,4-α-glucan branching enzyme preparation does not contain any diluents, carriers, preservatives, or stabilizers.

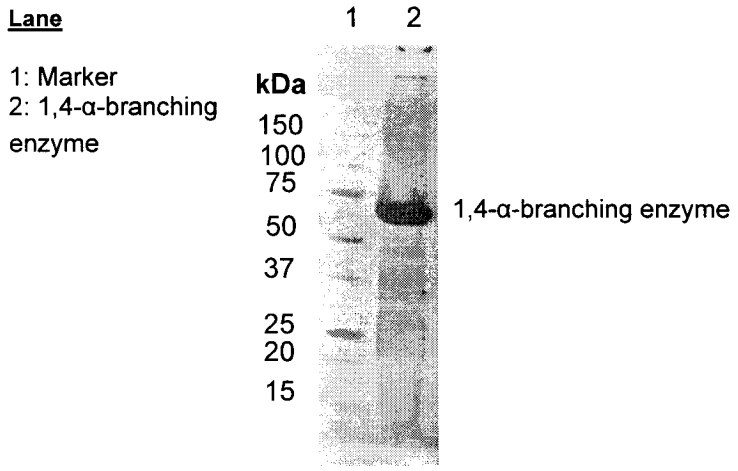
II.A.4.2 Presence of Secondary Enzyme Activities

The 1,4-α-glucan branching enzyme preparation does not possess any other enzymatic activities, including α-amylase or α-glucosidase. It should be noted that the manufacturing of

the 1,4-α-glucan branching enzyme preparation involves heat treatment, which would result in the inactivation of heat-labile proteins present in *B. subtilis.*

In addition, a 2-dimensional (2D) electrophoretic analysis of the enzyme preparation was performed *via* sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to visualize the complexity of the 1,4-α-glucan branching enzyme preparation, and the results are presented in Figure II.A.4.2-1 below. The 2D SDS-PAGE analysis demonstrate that 1,4-α-glucan branching enzyme, which has a molecular weight ranging from 62,000 to 66,000 g/mol, is the primary component of the enzyme preparation.

Figure II.A.4.2-1 2D SDS-PAGE Analysis of the 1,4-α-Glucan Branching Enzyme Preparation



II.A.4.3 Protein, Carbohydrate, Fat, Water, Ash, and Total Organic Solids Analyses

Three (3) non-consecutive lots of the 1,4-α-glucan branching enzyme preparation (Lot Nos. 090915, 060614, 05122-2) were analyzed for protein plus nucleic acid, carbohydrate, fat, water, ash, and total organic solids (TOS) content. The ash and water content ranged from 1.7 to 2.4% and 90.7 to 93.2%, respectively. Protein content was determined by the Kjeldahl method, and ranged from 2.2 to 3.5%. The Kjeldahl method is a quantitative measure of the total nitrogen content in organic compounds, and is used is used universally as an official method for crude protein content (NZIC, 1998; FCC, 2008; Nielsen, 2010). TOS content was determined by calculating the sum of the organic compounds present in the branching enzyme preparation. As the 1,4-α-glucan branching enzyme preparation is prepared from an organic source and no diluents or carriers are added to the preparation, the TOS content was determined to be 100% subtracted by ash and water content, and ranged from 4.4 to 6.9%. In addition, the fat content was determined to be <0.1% in all 3 lots. Reports of analysis are provided in Appendix C.

II.B Method of Manufacture

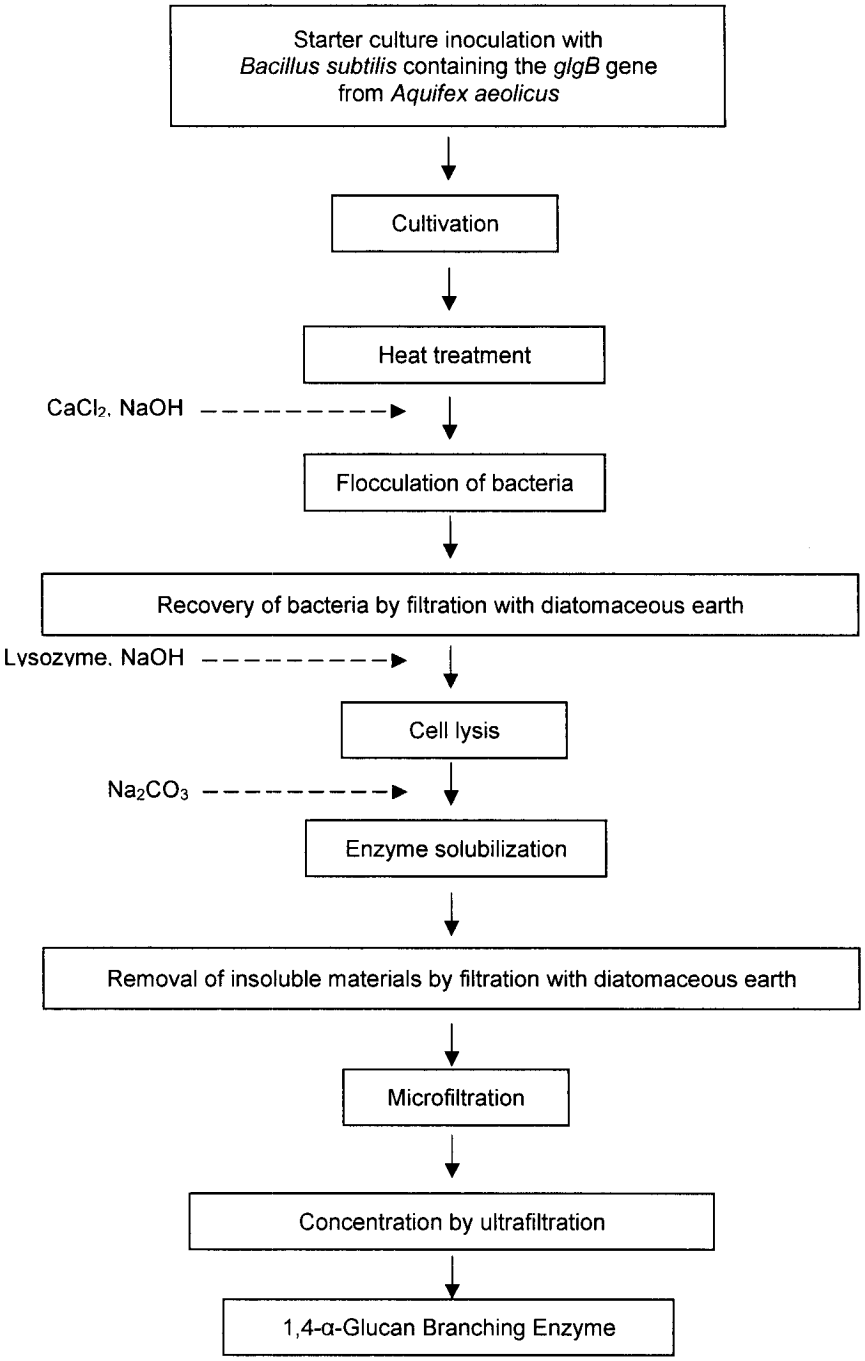
The1,4-α-glucan branching enzyme preparation is derived from a genetically modified strain of *B. subtilis* 168 containing the gene encoding the 1,4-α-glucan branching enzyme (*glgB*) of *A. aeolicus* VF5. The genetically modified production strain is produced using standard genetic modification techniques and the 1,4-α-glucan branching enzyme preparation is manufactured in accordance with cGMP using food-grade materials and using a quality-controlled fermentation and purification process. An overview of the manufacturing process of the 1,4-α-glucan branching enzyme preparation is presented in Figure II.B-1.

First, the starter culture is cultivated using food-grade liquid medium. The media used for culturing the production strain consist of liquid media composed of nutrient sources and materials that are commonly used in bacterial fermentation media. Sources of nutrients include peptones (protein hydrolysates) and yeast extract, which provide the amino acids and vitamins, respectively, required for bacterial growth. Dextrose, sucrose, sorbitol, or glycerol is added to the culture medium to provide the bacteria with a carbon/energy source. Manganese (II) is added to the medium in the form of the metal salt, manganese (II) chloride tetrahydrate ($MnCl_2 \cdot 4H_2O$) for bacterial nutrition. Sodium chloride, potassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4), and sodium hydroxide (NaOH) are used as buffering or pH control agents to adjust and maintain the pH of the culture medium for optimal growth conditions. All components of the culture medium meet food-grade specifications or are of adequate purity for food fermentation processes and are removed through extensive purification processes involved in the manufacture of the final product. Furthermore, all components of the culture medium and processing aids used in the manufacture of the branching enzyme preparation are used in compliance with appropriate U.S. federal regulations.

Secondly, the main liquid culture medium is inoculated with this starter culture. The main culture medium contains a food-grade defoaming agent to prevent excess foaming during cultivation. The bacteria are cultured, and NaOH is added to the culture medium for pH adjustment purposes. The culture is heat-treated prior to cell lysis, which serves to effectively denature any undesirable proteins. Following heat-treatment, the bacteria are lysed using lysozyme at neutral pH in order to release the intracellular branching enzyme. The branching enzyme is then solubilized using sodium carbonate (Na_2Co_3). The insoluble materials are removed from the branching enzyme solution by filtration with diatomaceous earth. The solution is further purified by microfiltration and is concentrated by ultrafiltration. Removal of the bacterial remnants is carried out at the filtration steps, preventing carry-over of the bacteria into the final enzyme preparation. Water is added to the branching enzyme preparation in order to adjust the enzymatic activity. No other diluents, carriers, stabilizers, or preservatives are added to the branching enzyme preparation.

Quality control steps are included throughout the fermentation and purification processes in order to ensure adherence with the established production process to produce a high quality and consistent product. Chemical and microbiological analyses are performed on the final enzyme preparation to ensure absence of heavy metal and microbiological contaminants as outlined in Section II.C.

Figure II.B-1 **Schematic Overview of the Manufacturing Process for the 1,4-α-Glucan Branching Enzyme Preparation**



II.C Specifications for Identity and Purity

The product specifications for the 1,4-α-glucan branching enzyme preparation are presented in Table II.C-1. The specifications for the 1,4-α-glucan branching enzyme preparation comply with current specifications for enzyme preparations of the Joint FAO/WHO Committee on Food Additives (JECFA) (JECFA, 2006), with the exception of the specification for the absence of anti-microbial activity. The reason for this non-inclusion is that the branching enzyme preparation has an alkaline pH of 10, which may inhibit bacterial growth, and thus, may lead to potential false positive results. The specifications for the 1,4-α-glucan branching enzyme preparation also meets the current specifications for enzyme preparations of the Food Chemicals Codex (FCC) (FCC, 2008), which do not include the test for the absence of anti-microbial activity. The product specifications include those for the physical appearance and activity of the branching enzyme, as well as specifications for potential chemical and microbiological impurities and presence of the production strain. Details of the methods of analysis are provided in Appendix B.

Table II.C-1 Specifications for the 1,4-α-Glucan Branching Enzyme Preparation

Specification Parameter	Specification Requirements	Method of Analysis
Appearance	Dark brownish liquid	Visual examination
Assay (Activity)	36,000 to 44,000 U/g	Branching enzyme activity assay[a,b]
Lead	Less than 5 ppm	AAS (FCC, 2008)[c]
Arsenic (as As_2O_3)	Less than 4 ppm	AAS (FCC, 2008)[c]
Total viable plate count	Less than 10,000 CFU/g	Standard agar plate method[b,d]
Coliforms	Negative	BGLB method[b,e]
Escherichia coli	Negative	Enrichment cultivation method[b,e]
Salmonella spp.	Negative	Enrichment cultivation method[b,f]
Production strain containing kanamycin resistance gene from plasmid pUB110	Negative	1. Plating method and test for branching enzyme activity[b,g] 2. Plating method and PCR for kanamycin resistance gene[b,g]

As_2O_3 = Arsenic oxide; AAS = atomic absorption spectrophotometry; BGLB = brilliant green lactose bile; CFU = colony forming unit; FCC = Food Chemical Codex; PCR = polymerase chain reaction

[a] Based on the ability of the enzyme to decrease the absorbance of amylose-iodine complexes.

[b] See Appendix B for a description of the method of analysis.

[c] FCC (2008). *Food Chemicals Codex, 6th edition.* Rockville (MD): United States Pharmacopeial Convention (USP).

[d] Limit of detection = 300 CFU/g.

[e] Limit of detection = 1 CFU/2.2 g.

[f] Limit of detection = 1 CFU/25g.

[g] Growth on kanamycin-containing medium, followed by visual examination of colony growth. The test is designed to confirm that the 1,4-α-glucan enzyme preparation contains no viable cells of the production strain. If colonies are detected, each colony is isolated and analyzed for branching enzyme activity. Detection of branching enzyme activity is considered to be indicative of a positive result for the presence of the production strain. Colonies with a positive result are then analyzed by PCR for the presence of the kanamycin-resistance gene. Branching enzyme preparations in which a positive result is obtained will not be used in food production. A positive [*B. subtilis* NCIMB12378 (pUAQ2)] and negative (*B. subtilis* NCIMB12378) control is included in the test. See Appendix B for a description of the method of analysis.

Analyses of 3 non-consecutive lots of the 1,4-α-glucan branching enzyme preparation confirm that the material produced is consistent and complies with the product specifications.

III. INTENDED TECHNICAL EFFECTS AND USE

III.A Technological Function of the 1,4-α-Glucan Branching Enzyme Preparation

The 1,4-α-glucan branching enzyme is intended for use in the production of modified food starch ingredients, including cyclic dextrin, highly branched produced from food-grade waxy corn starch. The 1,4-α-glucan branching enzyme preparation catalyzes the transfer of a terminal reducing glucose unit from the inner long α-(1,4) glucose B-chain of the amylopectin substrate to an acceptor glucose unit of the adjacent cluster *via* an α-(1,6) glucosidic bond (Takata *et al.*, 1994, 1996a,b, 2003).

III.B Intended Use of the 1,4-α-Glucan Branching Enzyme Preparation

The 1,4-α-glucan branching enzyme preparation is intended for use as a processing aid in the production of modified food starch ingredients, including cyclic dextrin, highly branched, with a typical use level of 500 U/g substrate. The branching enzyme is used only at minimum levels necessary to achieve the intended effect.

The cyclic dextrin, highly branched ingredient is intended for use in selected foods and beverages, including baked goods and baking mixes, beverages and beverage bases, breakfast cereals, tea, grain products, seasonings, milk and plant protein products, processed fruits and vegetables and fruit and vegetable juices, soft candy, and powdered soups.

III.C Residual Enzyme in the Final Food Ingredient

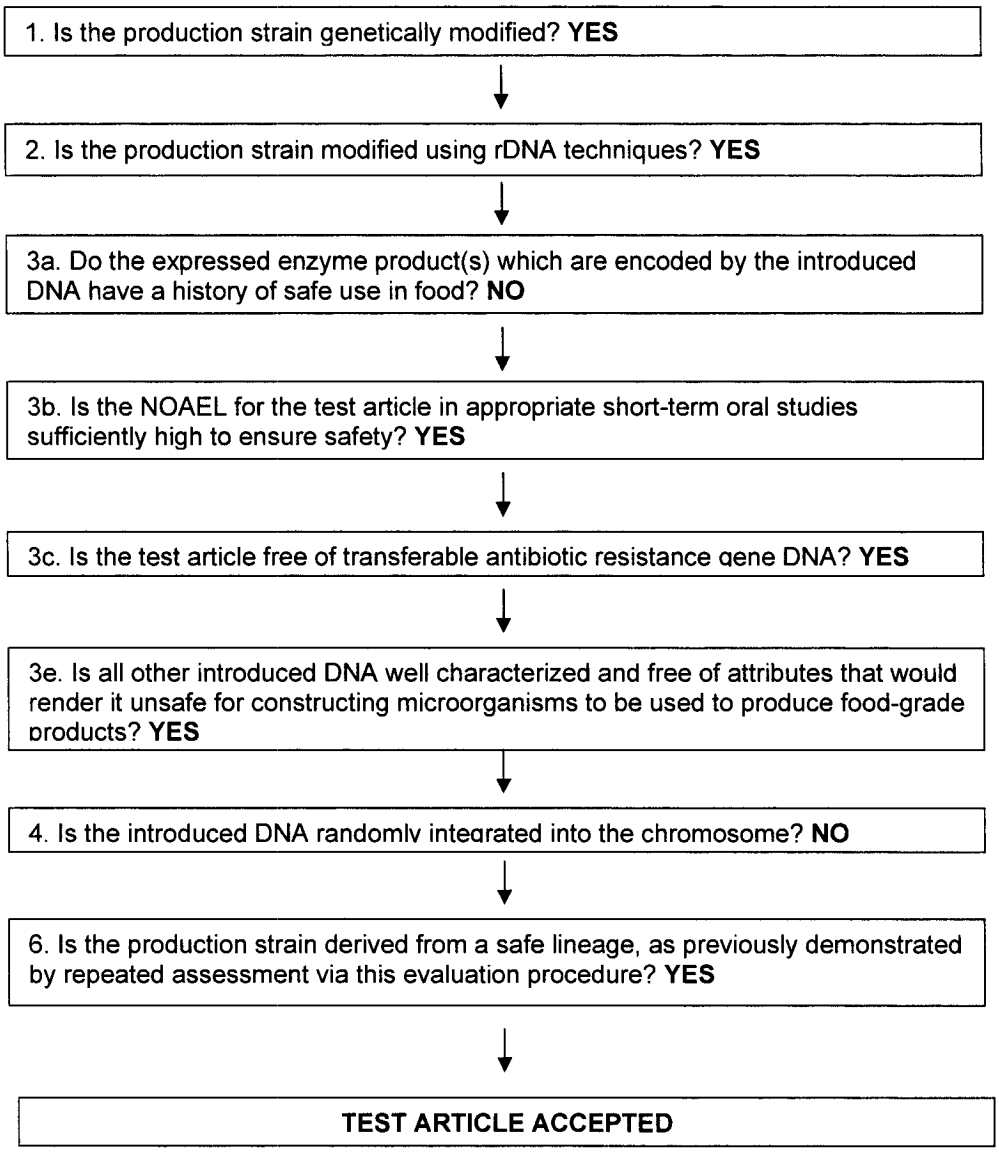
Considering that the 1,4-α-glucan branching enzyme preparation is used only as a processing aid, the enzyme itself is not added directly to food. Moreover, the manufacturing process for cyclic dextrin, highly branched incorporates steps to ensure inactivation and removal of the branching enzyme preparation. The branching enzyme preparation is denatured and inactivated by pH adjustment with hydrochloric acid and by heating at high temperatures. In addition, purification of the final ingredient by filtration through diatomaceous earth, perlite, and an ion-exchange resin ensures removal of the residual branching enzyme preparation. Thus, carry-over of the 1,4-α-glucan branching enzyme preparation into the final food ingredient, or into the final food product, is negligible to none. This is supported by analyses of 3 non-consecutive lots of the cyclic dextrin, highly-branched ingredient, which indicated that protein (as assessed by the Kjeldahl method) is not detectable in the final ingredient at a limit of detection of 0.1 g/100 g (0.1%).

IV. SAFETY OF 1,4-α-GLUCAN BRANCHING ENZYME PREPARATION

IV.A Safety of the Production Microorganism

The safety of the production microorganism was determined using the guidelines developed by Pariza and Foster (1983), Pariza and Johnson (2001), and IFBC (1990), presented in the form of a decision tree, and summarized in Figure IV.A-1. These guidelines are widely accepted by the scientific community and regulatory agencies as criteria for assessing the safety of microbial enzyme preparations used in foods. Using this decision tree, it is demonstrated that the production strain meets the safety criteria developed for microbial enzyme preparations.

Figure IV.A-1 Decision Tree for Determining the Safety of Genetically-Modified *Bacillus subtilis* for Use in Food



Modified from Pariza and Johnson (2001).

B. subtilis, including strain 168 which is used as the host strain in the production of the 1,4-α-glucan branching enzyme preparation, is a ubiquitous, aerobic, rod-shaped, spore-forming, non-pathogenic and non-toxigenic Gram-positive bacterium that is well-characterized and widely used in the production of food enzymes with a long history of safe use the food processing industry (de Boer and Diderichsen, 1991; Nakamura *et al.*, 1999; Olempska-Beer *et al.*, 2006). Of concern with all *Bacillus* species is the potential for producing *B. cereus* enterotoxins; however, *B. subtilis* has been shown to not contain genes with homology to those encoding for the known *B. cereus* enterotoxins and to further not produce such toxins (de Boer

and Diderichsen, 1991; U.S. EPA, 1997; Pedersen *et al.*, 2002; Olempska-Beer *et al.*, 2006). As a result *B. subtilis* has been granted Qualified Presumption of Safety (QPS) status in the EU (EFSA, 2008). Although QPS status is exclusive of genetically modified microorganisms, *B. subtilis* 168 is the progenitor of many recombinant *B. subtilis* strains with a history of safe use as sources of food enzymes (Olempska-Beer *et al.*, 2006). Thus, the use of *B. subtilis* 168 is considered safe for the derivation of genetically-modified strain lineages intended for use in the production of food enzymes.

The source microorganism, *A. aeolicus*, is a microaerophilic, hyperthermophilic, rod-shaped, Gram-negative bacterium that is found to occur in hyperthermophilic environments (Deckert *et al.*, 1998; Huber and Stetter, 2001). A search of the scientific literature has not revealed evidence that this bacterium harbors pathogenic or toxigenic potential. Moreover, the DNA sequence encoding 1,4-α-glucan branching enzyme from *A. aeolicus* VF5 does not encode for a product with sequence similarity to principal bacterially-produced toxins. Instead, expression of the DNA insert, when incorporated into *B. subtilis*, solely produces the desired product (*i.e.*, 1,4-α-glucan branching enzyme). Therefore, *A. aeolicus* VF5 can be considered as a safe source for the 1,4-α-glucan branching enzyme.

Introduction of the vector expression system, pUAQ2, consisting of the vector pUB110 and the DNA sequence for the *glgB* gene of *A. aeolicus* VF5, does not have any impact on the non-toxigenic and non-pathogenic status of the host microorganism. The DNA insert introduced encodes only for the desired protein product (*i.e.*, 1,4-α-glucan branching enzyme), which does not have sequence similarity to principal bacterially-produced toxins. This was determined using the National Center for Biotechnology Information's (NCBI) protein database using the Protein Basic Local Alignment Search Tool (Protein BLAST) program. In addition, vector pUB110 is a well-characterized vector that is documented to be safe for introducing new genetic material into *B. subtilis* for the purpose of producing food enzymes (IFBC, 1990; Olempska-Beer *et al.*, 2006). Although pUB110 harbors an antibiotic (kanamycin) resistance gene, the enzyme product itself is free of transferable antibiotic resistance gene DNA, since such resistance genes can be considered to be stably associated with the vector (IFBC, 1990). Moreover, the final enzyme preparation is tested for the presence of the production strain containing the kanamycin resistance gene as indicated in Section II.C. An enzyme preparation in which a positive result is obtained is not used in food production. The presence of the antibiotic resistance gene also is of no further safety concern, considering that the production strain does not enter the final food product and is not deliberately released into the environment; therefore, transfer of the antibiotic resistance gene to other organisms is not likely to occur.

IV.B Toxicological Studies with the 1,4-α-Glucan Branching Enzyme Preparation

IV.B.1 Mutagenicity and Genotoxicity Studies

A bacterial reverse mutation test (Ames test) was conducted to assess the potential mutagenicity of the 1,4-α-glucan branching enzyme preparation at concentrations of 312, 625, 1,250, 2,500, or 5,000 µg/plate in *Salmonella typhimurium* TA98, TA100, TA1535, and TA1537 and *Escherichia coli* WP2*uvr*A (Choi *et al.*, 2009). This study was conducted in compliance with the Organisation of Economic Co-operation and Development (OECD) principles of Good Laboratory Practice (GLP) (OECD, 1998a), and in accordance with OECD Test Guideline No. 471 (OECD, 1997a). The water vehicle served as a negative control for all strains. One of the following compounds was employed as a positive control for assays conducted in the absence of S9 metabolic activation: sodium azide, 9-aminoacridine (9AA), 2-nitrofluorene, N-ethyl-N-nitrosourea, or 1-methyl-3-nitro-1-nitrosoguanidine. For assays conducted in the presence of metabolic activation, 2-aminoanthracene (2-AA) or benzo[a]pyrene was employed as the positive control. Bacterial strains were treated with the branching enzyme preparation at concentrations of 62, 185, 556, 1,667, or 5,000 µg/plate. No inhibition of bacterial growth was observed for any of the strains treated with the branching enzyme preparation in the presence or absence of S9 metabolic activation compared to the negative control. Treatment with the branching enzyme preparation did not result in more than a 2-fold or dose-dependent increase in the mean number of revertant colonies compared with the negative control at any concentration, with or without metabolic activation. Positive control agents substantially induced the number of revertant colonies compared to the negative control, confirming the sensitivity of the assay. Based on these findings, the 1,4-α-glucan branching enzyme preparation is non-mutagenic as assessed in the Ames test.

The genotoxic potential of the 1,4-α-glucan branching enzyme preparation was further evaluated in a chromosomal aberration assay in cultured Chinese hamster ovary (CHO) cells with and without metabolic activation (Choi *et al.*, 2009). This study was conducted in compliance with the OECD principles of GLP (OECD, 1998a) and in accordance with OECD Test Guideline No. 473 (OECD, 1997b). The culture medium served as the negative control. Mitomycin C [in the absence of metabolic activation (S9)] and cyclophosphamide (in the presence of S9) were employed as positive controls. In the first experiment, cells were treated with the enzyme preparation at final concentrations of 10, 20, 39, 78, 156, 313, 625, 1,250, 2,500, or 5,000 µg/mL. Cells were either pulse-treated or continuously-treated with the branching enzyme preparation. Cells co-incubated with the enzyme preparation and S9 were only subjected to pulse treatment. Cells were arrested in metaphase and blindly scored to determine the mitotic index (percentage of cells undergoing mitosis) as a measurement of cytotoxicity. The enzyme preparation did not reduce the mitotic index of CHO cells at any concentration (up to 5,000 µg/mL) compared to the negative control in either the first or second experiment following either pulse or continuous treatment. Additionally, the enzyme preparation

did not significantly increase the number of aberrant cells at any concentration compared to the negative control in any of the assays. In both experiments, positive controls significantly induced the incidence of structural chromosomal aberrations compared to the negative control. Therefore, the 1,4-α-glucan branching enzyme preparation is non-genotoxic as assessed in the chromosomal aberration assay.

IV.B.2 Repeated-Dose Studies

A 13-week oral toxicity study was conducted to assess the potential toxicity of the 1,4-α-glucan branching enzyme preparation. This study was conducted in compliance with the Ministry of Health and Welfare (MHW) principles of GLP, and also was performed according to the Japanese guidelines for repeated dose toxicity studies and OECD Test Guideline No. 408 (OECD, 1998b). In this study, 5-week-old Sprague-Dawley [Crl:CD(SD)] rats (10/sex/group) were administered the enzyme preparation at volumes of 1.7 (low-dose), 5 (mid-dose), or 15 (high-dose) mL/kg body weight/day by oral gavage (Choi *et al.*, 2009). Control animals were administered 15 mL of vehicle (water). The administered test article was reported to have a TOS value of 60 mg/mL and therefore, the administered doses were equivalent to levels of 102, 300, and 900 mg TOS/kg body weight/day, respectively, of the enzyme preparation. The animals were observed daily for general condition and mortality before and after test article administration. Detailed clinical observations were recorded the day before the first administration and once a week thereafter. Body weights and food consumption also were measured the day before the first administration, as well as within the first 2 days of the study and once a week thereafter. Clinical observations, body weights, and food consumption also were recorded on the day of necropsy. An ophthalmological examination was performed on 5 animals from each group in Week 13 of the study. In Week 13, urine was collected over a period of 18 hours and urinalysis performed. Hematological and clinical chemistry analyses were conducted on blood collected on the day following the final administration day. All animals were necropsied on the day following the final administration day and organs were weighed and examined macroscopically. Selected tissues were further examined microscopically.

All test and control group animals survived the course of the study and appeared in good general condition, with the exception of tooth loss in 1 male of the mid-dose group, which occurred on Day 56 of the study. This event was likely not compound-related, but rather a random occurrence, as tooth loss was not observed at the highest dose and only occurred in a single animal of one gender. Body weights throughout the course of the study did not differ significantly between any of the test groups and the control group. Variations in food consumption were limited to a transient decrease in mid-dose males on Day 70 and an increase in females of the high-dose group on Day 14. The significant reduction in mid-dose males was attributed to decreased food intake by the animal exhibiting tooth loss as no significant differences in food intake between the test and control groups were reported following the exclusion of this animal from statistical analysis. The alterations in food intake observed in high-

dose females were likely not compound-related as dose- and time-dependent trends were not observed. Upon ophthalmological examination, 1 male of the low-dose group (exhibited meandering of the left artery. This was not considered to be compound-related as this finding did not occur in any other animal administered the enzyme preparation. No other ophthalmological variations were observed in test or control animals. Urinalysis results showed a significant, but less than 2-fold increase, in the concentration and total excretion of sodium in male and female rats of the high-dose group compared to controls. All other urinalysis results were similar between test and control animals. Hematology revealed the following significant alterations in test groups compared to controls: in females, decreases in red blood cell count at all doses, hemoglobin concentration and hematocrit at the mid- and high-dose, and platelet count at the low- and high-dose; in males, an increase in prothrombin time and a decrease in activated partial thromboplastin time at the mid- and high-dose. All other hematology results were similar between test and control animals. Clinical chemistry results indicated significant increases in total cholesterol and β-globulin levels in males of the mid-dose group compared to control males. Results of all other serum chemistry parameters did not differ significantly between test and control groups and no deviations from control values were observed in females administered the enzyme preparation.

Significant increases in the absolute and relative uterus weights were observed in female animals of the low-dose group compared to controls. All other absolute and relative organ weights were similar between test and control groups. Macroscopic variations were limited to the occurrence of discoloration of the liver in rats of all test and control groups. Discoloration was observed in 2 males and 1 female in the control group, 5 males in the low-dose group, 1 female in the mid-dose group, and 3 males and 2 females in the high-dose group. In addition, a nodule, 8 mm in diameter, was detected in the left kidney in 1 control female. No other gross organ abnormalities were observed in control or test animals. Livers from all groups of animals were subject to further histopathological examination. Microscopic findings observed in the liver included perilobular hepatocellular vacuolation, microgranuloma, and bile duct proliferation. However, the incidences of these microscopic finding did not differ significantly between control and test animals. The frequencies of all other histopathological observations were comparable between the control and test groups and were thus considered not related to the administration of the enzyme preparation.

Changes in urinalysis, clinical chemistry, and hematology parameters, as well as in organ weights, observed in test groups compared to controls were not considered to be of toxicological significance. Specifically, increases in urinary sodium excretion in the high-dose groups were not accompanied by compound-related changes in blood electrolyte concentration or by increases in blood urea nitrogen or creatinine levels that would be indicative of impaired renal function or by histopathological variations in the kidneys. Increases in serum chemistry parameters in males of the mid-dose group and increases in uterine weights in females of the low-dose group were not observed at higher doses and were not accompanied by

histopathological abnormalities. In addition, changes in hematology parameters upon exposure to the branching enzyme were not considered to be toxicologically significant as alterations only occurred in one gender, were not dose-dependent, and were not associated with compound-related histopathological abnormalities.

The frequency of the macroscopic and microscopic changes observed in the liver did not differ significantly between test and control groups. No compound-related abnormalities were noted in other tissues. Therefore, the investigators concluded that under the conditions of this study, the no-observed-adverse-effect level (NOAEL) for the 1,4-α-glucan branching enzyme preparation is 15 mL/kg body weight/day (approximately 900 mg TOS/kg body weight/day) in experimental rats, the highest dose tested.

IV.C Safety Evaluations

Recently, JECFA evaluated the safety of a 1,4-α-glucan branching enzyme preparation derived from *B. subtilis* expressing the branching enzyme gene from *Rhodothermus obamensis*. A toxicological monograph was prepared summarizing the technical and toxicological data. Included in the toxicological data were a 90-day oral (gavage) toxicity study in rats, a bacterial reverse mutation test, and an *in vitro* mammalian cell micronucleus test all performed according to OECD test guidelines (JECFA, 2010a). Based on the results of the 90-day study, a NOAEL of 769 mg TOS/kg body weight/day was reported for the enzyme preparation. In addition, negative results were observed in the mutagenicity/genotoxicity studies. The Committee allocated an acceptable daily intake (ADI) of not specified[3] for the 1,4-α-glucan branching enzyme preparation when used in the specified applications and in accordance with Good Manufacturing Practices (GMP) (JECFA, 2010a,b). Although the evaluation was specific to the 1,4-α-glucan branching enzyme preparation derived from *B. subtilis* expressing the branching enzyme gene from *R. obamensis*, the results of the toxicological studies and the resultant evaluation support the safe use of 1,4-α-D-glucan branching enzyme preparations produced using safe bacterial production strains. Furthermore, this same enzyme preparation was the subject of a GRAS exemption claim (GRN 000274, U.S. FDA, 2009), and which received a no questions letter from FDA regarding the self-GRAS determination.

[3] A term applicable to a food substance of very low toxicity which, on the basis of the available data (chemical, biochemical, toxicological, and other), the total dietary intake of the substance arising from its use at the levels necessary to achieve the desired effect and from its acceptable background in food does not, in the opinion of JECFA, represent a hazard to health (WHO, 1987).

IV.D Additional Safety Considerations

IV.D.1 Potential Allergenicity

A search conducted of the NCBI's protein database using the Protein BLAST program failed to identify any protein sequence similarity between 1,4-α-glucan branching enzyme and known bacterially-produced toxins or known food allergens. An additional search was conducted according to the approach outlined by the FAO/WHO (FAO/WHO, 2001) using the Food Allergy Research and Resource Program (FARRP) Protein AllergenOnline Database (version 10.0, available at http://www.allergenonline.org/), which contains a comprehensive list of putative allergenic proteins developed *via* a peer reviewed process for the purpose of evaluating food safety. In accordance with the FAO/WHO guideline, the database was searched using a sliding window of 80-amino acid sequences derived from the full-length 1,4-α-glucan branching enzyme protein sequence as presented in Section II.A.1. Significant homology is defined as an identity match of greater than 35%, and in such instances, cross-reactivity with the known allergen must be considered. Using this search strategy, no identity matches of greater than 35% were identified. The FAO/WHO also notes that cross-reactivity with a known allergen must be considered when 6 contiguous amino acids are identified. The AllergenOnline sequence search tools do not include a function to search for 6 contiguous amino acids, but do include a function for searching for 8 contiguous amino acids. Using this tool, no exact matches were identified. See Appendix D for the search results.

Although the major known milk allergen, casein, is used as a component of the fermentation medium, this would be removed during the purification processes outlined in Section II.B. Moreover, the manufacturing process for the cyclic dextrin, highly branched ingredient incorporates steps to ensure inactivation and removal of the enzyme preparation; thus, it is not expected that the enzyme preparation, when used as a food processing aid, would cause allergic reactions. In support of this, analysis of the final ingredients demonstrated the absence of protein residues (limit of detection of 0.1% for total protein), indicating that casein is not present in the final ingredients that are added to food. Furthermore, no adverse events, including those of an allergenic nature, have been reported from the consumption of a commercial cyclic dextrin, highly branched in Japan.

IV.E Estimated Human Exposure

The 1,4-α-glucan branching enzyme preparation is intended for use as a processing aid in the production of modified food starch ingredients, and is not added directly to food. As a worst-case scenario, the following assumptions were made to estimate the human exposure to the branching enzyme preparation:

1) All of the processed foods and beverages containing the modified food starch ingredient (*i.e.,* cyclic dextrin, highly branched) produced using the 1,4-α-glucan

branching enzyme preparation as a processing aid, used the typical use-level of 500 U/g substrate;

2) All TOS in the 1,4-α-glucan branching enzyme preparation remains in the final modified food starch ingredient; and
3) The 90th percentile all-user intake of cyclic dextrin, highly branched by the total U.S. population is estimated to be 48 g/person/day or 1,011 mg/kg body weight/day.

The 1,4-α-glucan branching enzyme preparation contains approximately 7,414 U/mg TOS (the average of 3 batches), and is being used as a processing aid in the production of the food ingredient cyclic dextrin, highly branched at a typical use-level of 500 U/g substrate, which corresponds to:

500 U/g substrate x 1 mg TOS/7,414 U = 0.07 mg TOS/g substrate

The 90th percentile all-use intake of cyclic dextrin, highly branched by the total U.S. population is estimated to be 48 g/person/day or 1,011 mg/kg body weight/day. Based on the assumption that the entire enzyme preparation used in the manufacturing of cyclic dextrin, highly branched is retained in the final product, the maximum estimated daily intake of the 1,4-α-glucan branching enzyme preparation would be:

0.07 mg TOS/g substrate x 1,011 mg/kg body weight/day = 0.07 mg TOS/kg body weight/day

The NOAEL of the 1,4-α-glucan branching enzyme preparation was determined to be 15 mL/kg body weight/day (approximately 900 mg TOS/kg body weight/day) in experimental rats, the highest dose tested, in a 90-day repeated-dose study (see Section IV.A.2). On a body weight basis, this is approximately 12,857 times greater than the amount of enzyme that would be expected to be consumed by humans from the proposed use of cyclic dextrin, highly branched in food, respectively, under the "worst-case scenario".

V. SUMMARY AND BASIS FOR GRAS CONCLUSION

1,4-α-Glucan branching enzyme preparation [(1→4)-α-D-glucan:(1→4)-α-D-glucan 6-α-D-[(1→4)-α-D-glucano]-transferase); EC 2.4.1.18; CAS No. 9001-97-2] is produced by genetically-modified *Bacillus subtilis* 168 expressing the 1,4-α-glucan branching enzyme structural gene *glgB* from *Aquifex aeolicus* VF5. Genetic modification of *B. subtilis* was performed using standard techniques and the branching enzyme preparation is manufactured according to cGMP, using food-grade materials and a quality-controlled fermentation and purification process. The final 1,4-α-glucan branching enzyme preparation meets appropriate food-grade specifications and complies with the current specifications for enzyme preparations laid down by JECFA and in the FCC. Lot samples are routinely assayed to verify compliance with the specifications, including those for chemical impurities and microbiological contaminants, and

non-consecutive batch analyses confirm the consistency of the final product and reproducibility of the manufacturing process.

The 1,4-α-glucan branching enzyme preparation from genetically-modified *B. subtilis* 168 expressing the 1,4-α-glucan branching enzyme gene from *A. aeolicus* VF5 is specifically intended for use in the production of modified food starch ingredients, including highly-branched cyclic dextrins (also known as cyclic dextrin, highly branched) produced from food-grade waxy corn starch, at a typical use level of 500 U/g substrate. The cyclic dextrin, highly branched ingredient is intended for use in a variety of traditional foods and beverages. As the branching enzyme preparation is used only as a processing aid, it will not be added directly to food, and the manufacturing processes for the final food ingredients incorporate steps to ensure inactivation and removal of the branching enzyme preparation. Thus, carry-over of the branching enzyme preparation into the final food ingredient, or into the final food product, is negligible to none.

The safety of the 1,4-α-glucan branching enzyme preparation was supported using safety guidelines for microbial enzyme preparations developed by Pariza and Foster (1983), Pariza and Johnson (2001), and the IFBC (1990). The safety evaluation took into consideration the toxigenic and pathogenic potential of the production strain, including the following elements: the host organism; the donor organism; and the DNA to be introduced, including the DNA insert (*i.e.*, the gene of interest) and the vector. *B. subtilis* is a non-toxigenic and non-pathogenic Gram-positive bacteria considered safe for use in food processing. In addition, *B. subtilis* strain 168 is the progenitor of many recombinant *B. subtilis* strains that have been used as sources of enzymes utilized in food processing (Olempska-Beer *et al.*, 2006). Thus, *B. subtilis* 168 also is considered safe for use as a host microorganism. The transfer of the *glgB* gene from *A. aeolicus* strain VF5, a Gram-negative bacterium, is considered safe in the manufacture of the branching enzyme, as no evidence indicates that the strain harbors pathogenic or toxigenic potential. The DNA encoding the branching enzyme (the *glgB* gene) from *A. aeolicus* VF5 does not encode for a product with sequence similarity to principal bacterially-produced toxins, such as *B. cereus* toxin (Choi *et al.*, 2009). Instead, expression of the DNA inserts, incorporated into *B. subtilis*, solely produce the desired product (*i.e.*, the 1,4-α-glucan branching enzyme preparation). This information, in addition to the GRAS exemption claim for 1,4-α-glucan branching enzyme derived from a different production strain (GRN 000274, U.S. FDA, 2009), supports the safety of the DNA inserts for use in food processing. Furthermore, the vector carrying the gene of interest, pUB110, is a well-characterized safe vector for introducing new genetic material in *B. subtilis* for the purpose of producing food enzymes (IFBC, 1990; Olempska-Beer *et al.*, 2006). Thus, the 1,4-α-glucan branching enzyme preparation is considered safe for use in food production.

The safety of the 1,4-α-glucan branching enzyme preparation was further assessed in standard toxicological tests, specifically in a subchronic oral toxicity study and in *in vitro* mutagenicity/

genotoxicity assays. In a subchronic 13-week toxicity study, repeated oral administration of the branching enzyme preparation at doses of up to 15 mL/kg body weight/day (equivalent to 900 mg TOS/kg body weight/day) in rats did not result in compound-related adverse effects with respect to clinical signs of toxicity, body weight gain, food consumption, hematology, clinical chemistry, urinalysis, organ weights, or gross and microscopic findings (Choi *et al.*, 2009). Under the conditions of these studies, a NOAEL of 900 mg TOS/kg body weight/day, the highest dose tested, was determined for the 1,4-α-glucan branching enzyme preparation. The 1,4-α-glucan branching enzyme preparation also was determined to be non-genotoxic in the standard bacterial reverse mutation test conducted in a battery of *S. typhimurium* strains and *E. coli*, as well as in the chromosomal aberration assay conducted in CHO cells at concentrations of up to 5,000 µg/plate (Choi *et al.*, 2009). Collectively, the results of these studies indicate that the use of the 1,4-α-glucan branching enzyme preparation as a processing aid in food production is not expected to produce adverse effects in humans.

An Expert Panel convened on behalf of Ezaki, independently and collectively, critically evaluated the data and information summarized above, and concluded that the intended uses of the 1,4-α-glucan branching enzyme preparation from genetically-modified *B. subtilis* 168 expressing the 1,4-α-glucan branching enzyme gene from *A. aeolicus* VF5 for use as a processing aid in the production of cyclic dextrin, highly branched, produced consistently with cGMP and meeting appropriate food grade specifications, are safe and suitable.

The Expert Panel likewise unanimously concluded that the intended uses of 1,4-α-glucan branching enzyme preparation from genetically-modified *B. subtilis* 168 expressing the 1,4-α-glucan branching enzyme gene from *A. aeolicus* VF5 are GRAS based on scientific procedures and as such, it is Ezaki's opinion that other qualified and competent scientists reviewing the same publicly available toxicological and safety information would reach the same conclusion. Therefore, Ezaki has concluded that 1,4-α-glucan branching enzyme derived from genetically-modified *B. subtilis* 168 expressing the 1,4-α-glucan branching enzyme gene from *A. aeolicus* VF5 is GRAS based on scientific procedures for its intended use as a processing aid in the production of the modified food starch ingredient, cyclic dextrin, highly branched, and thus may be marketed and sold for the uses designated above in the U.S. without the promulgation of a food additive regulation under Title 21 of the CFR.

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APPENDIX A

APPENDIX A

EXPERT PANEL STATEMENT

Expert Panel Consensus Report Regarding the Generally Recognized as Safe (GRAS) Status of 1,4-α-D Glucan Branching Enzyme Preparations Derived from *Bacillus stearothermophilus* or Genetically-Modified *B. subtilis* with the Branching Enzyme Gene from *B. stearothermophilus* or *Aquifex Aeolicus* for Use as a Processing Aid in the Manufacture of Cyclic Dextrin, Highly-Branched and Enzymatically-Synthesized Glycogen

July 14, 2010

INTRODUCTION

At the request of Ezaki Glico Co., Ltd. (Ezaki Glico), an Expert Panel (the "Panel") of independent scientists, qualified by their relevant national and international experience and scientific training to evaluate the safety of food ingredients, was specially convened to conduct a critical and comprehensive evaluation of the available pertinent data and information, and determine whether, under the conditions of intended use as a food processing aid, 1,4-α-D-glucan branching enzyme [1,4-α-D-glucan: 1,4-α-D-glucan 6-α-D-(1,4-α-D-glucano)-transferase; EC 2.4.1.18; CAS No. 9001-97-2] (herein referred to as branching enzyme) would be Generally Recognized as Safe (GRAS) based on scientific procedures. The Panel consisted of the below-signed qualified scientific experts: Dr. Joseph F. Borzelleca (Virginia Commonwealth University School of Medicine) and Dr. William J. Waddell (University of Louisville School of Medicine).

The Panel, independently and collectively, critically examined a comprehensive package of scientific information and data compiled from the literature and other published sources through April 2010 by Cantox Health Sciences International (Cantox). In addition, the Panel evaluated other information deemed appropriate or necessary, including data and information provided by Ezaki Glico. The information evaluated by the Panel included details pertaining to the method of manufacture and product specifications, supporting analytical data, intended uses in food processing, and a comprehensive assessment of the available scientific literature pertaining to the safety of the branching enzyme.

Following independent, critical evaluation of such data and information, the Panel unanimously concluded that the intended uses in food processing, described herein, for the branching enzyme, meeting appropriate food-grade specifications as described in the supporting dossiers [Documentation Supporting the Evaluation of Cyclic Dextrin, Highly Branched as Generally Recognized as Safe (GRAS) for Use in Traditional Food Products and Further Documentation Supporting the Evaluation of 1,4-α-D-Glucan Branching Enzyme as Generally Recognized as Safe (GRAS) for Use in Food Processing] and manufactured consistent with current Good Manufacturing Practice (cGMP), are GRAS based on scientific procedures. A summary of the basis for the Panel's conclusion is provided below.

SUMMARY AND BASIS FOR GRAS

Ezaki Glico intends to apply the branching enzyme in the processing of food-grade hydrolyzed waxy corn starch and food-grade dextrin for the production of cyclic dextrin, highly branched and enzymatically-synthesized glycogen ingredients, respectively. The branching enzyme catalyzes the transfer of a terminal reducing glucose unit from the inner long α-(1,4) glucose B-chain of the hydrolyzed starch/dextrin substrate to an acceptor glucose unit of the adjacent cluster *via* a α-(1,6) glucosidic bond (Takata *et al.*, 1994, 1996a,b, 2003). The reaction, therefore, consists of a cyclization reaction resultant of intramolecular transglycosylation.

The branching enzyme is encoded by the *glgB* gene and is derived from one of the following three bacterial sources: non-genetically-modified *Bacillus stearothermophilus* strain TRBE14, *Bacillus subtilis* strain 168 genetically-modified to express the *glgB* gene from *B. stearothermophilus* TRBE14, and *B. subtilis* strain 168 genetically-modified to express the *glgB* gene from *Aquifex aeolicus*. The branching enzyme preparations produced using these bacterial sources are referred to as BE, BE-01, and BE-02, respectively. To produce BE-01, the *glgB* gene was cloned from the genomic DNA of *B. stearothermophilus* TRBE14 as described by Takata *et al.* (1994). To produce BE-02, the *glgB* gene from *A. aeolicus* was chemically synthesized as described by Takata *et al.* (2003). Each DNA fragment was then inserted into vector pUB110 using standard molecular cloning techniques. The resultant plasmids were transfected into *B. subtilis*. To produce the branching enzyme preparations, the bacteria are cultivated by liquid culture using food-grade materials as components of the culture medium. Following cultivation, the bacteria are lysed using lysozyme (from egg whites), allowing for the isolation of the branching enzyme. The branching enzyme preparation is then purified by filtration to remove insoluble materials and is further purified by microfiltration and concentrated by ultrafiltration. All processing aids used in the production of the branching enzyme preparations are of food-grade quality and the preparations are manufactured consistent with cGMP and meet appropriate food-grade specifications. Lot samples (at least 3 non-sequential lots) are routinely analyzed to ensure their compliance with the product specifications.

The final branching enzyme preparations are dark-brown solutions. The enzyme activity of the enzyme preparations is at least 10,000 U/mL and is highly specific to branching enzyme activity. The catalytic residues consist of two aspartic acid (Asp) residues and one glutamic acid (Glu) residue, which have also been identified as important for substrate-binding. The branching enzyme preparations are intended for use as processing aids in food production, and therefore, are not intended to be added directly to food. Moreover, the manufacturing processes for the final food ingredients incorporate steps to ensure removal of the bacteria and inactivation and removal of the branching enzyme. Specifically, carry-over of the bacteria into the final ingredients is prevented at the enzyme production stage, by lysing and removing the bacteria from the enzyme preparations. In addition, the branching enzyme is denatured and inactivated by pH adjustment with hydrochloric acid and by high temperature heating. Thus, only a purified enzyme filtrate is added to the starting material for the synthesis of cyclic dextrin, highly-

branched and enzymatically-synthesized glycogen. Purification of these final ingredients by filtration through diatomaceous earth, perlite, and an ion-exchange resin also ensures removal of the production enzymes and any residual particles. Thus, carry-over of the branching enzyme into the final ingredients and into the final food products is negligible. In support of the negligible carry-over of the branching enzyme, analyses of 3 non-consecutive lots of each of the cyclic dextrin, highly-branched and enzymatically-synthesized glycogen ingredients indicate that protein (as assessed by the Kjeldahl method) is not detectable in the final ingredients at a limit of detection of 0.1 g/100 g (0.1%).

The safety assessment of the branching enzyme preparations included an evaluation of the production strains and the genetic material introduced for genetically-modified strains, as well as an evaluation of traditional toxicological studies performed on the branching enzymes. The production strain for BE is *B. stearothermophilus* TRBE14 and that for BE-01 and BE-02 is genetically-modified *B. subtilis* 168. Both *B. stearothermophilus* TRBE14 and *B. subtilis* 168 lack toxigenic potential, have a history of safe use in food processing, and have been determined to be derived from a safe lineage (de Boer and Diderichsen, 1991; U.S. EPA, 1997; Pedersen *et al.*, 2002; Olempska-Beer *et al.*, 2006). In the case of the genetically-modified strains, the pUB110 vector carrying the *glgB* gene encoding the branching enzyme from *B. stearothermophilus* (BE-01) or *Aquifex aelicus* (BE-02) is a well characterized vector that has been determined to be safe for introducing new genetic material in *B. subtilis* for the purpose of producing enzymes used in the manufacture of food products (IFBC, 1990; Olempska-Beer *et al.*, 2006).

The safety of each branching enzyme preparation (BE, BE-01, and BE-02) has been evaluated in acute and/or subchronic toxicity studies, and/or *in vitro* mutagenicity assays. BE was not acutely toxic; the acute oral LD_{50} in Sprague-Dawley rats was greater than 2,000 mg/kg body weight (Choi *et al.*, 2009a). In subchronic 13-week, oral toxicity studies, repeated oral administration of BE-01 or BE-02 at doses of up to 15 mL/kg body weight/day (870 mg/kg body weight/day) in rats did not result in compound-related adverse effects with respect to clinical signs of toxicity, body weight gain, food consumption, hematology, clinical chemistry, urinalysis, organ weights, or gross and microscopic findings (Choi *et al.*, 2009b). Under the conditions of these studies, a no-observed-adverse-effect level of 870 mg/kg body weight/day, the highest dose tested, was determined for both BE-01 and BE-02. BE was non-mutagenic in a mouse lymphoma assay conducted in L5178Y $TK^{+/-}$ cells at concentrations of up to 5,000 µg/mL (Choi *et al.*, 2009a). BE-01 and BE-02 also were non-mutagenic in the Ames assay conducted in *Salmonella typhimurium* and *Escherichia coli* as well as in the chromosomal aberration assay conducted in CHO cells at concentrations of up to 5,000 µg/plate (Choi *et al.*, 2009b). The results of these *in vivo* and *in vitro* studies indicate that the use of BE, BE-01, or BE-02 in food production is not expected to be associated with any adverse effects.

CONCLUSION

We, the Expert Panel, have, independently and collectively, critically evaluated the data and information summarized above and conclude that the intended uses of the 1,4-α-D-glucan branching enzyme preparations derived from non-genetically modified *Bacillus stearothermophilus* TRBE14 (BE) or genetically-modified *Bacillus subtilis* 168 (BE-01 and BE-2) for use in the production of cyclic dextrin, highly branched and enzymatically-synthesized glycogen, meeting appropriate food-grade specifications presented in the supporting dossiers [Documentation Supporting the Evaluation of Cyclic Dextrin, Highly Branched as Generally Recognized as Safe (GRAS) for Use in Traditional Food Products] and [Additional Documentation Supporting the Evaluation of 1,4-α-Glucan Branching Enzyme Preparations as Generally Recognized as Safe (GRAS) for Use in Food Production] and produced consistent with current Good Manufacturing Practices (cGMP), are safe and suitable.

We further conclude that the intended uses of the 1,4-α-D-glucan branching enzyme preparations derived from non-genetically modified *Bacillus stearothermophilus* TRBE14 (BE) or genetically-modified *Bacillus subtilis* 168 (BE-01 and BE-02) for use in the production of cyclic dextrin, highly branched and enzymatically-synthesized glycogen, meeting appropriate food-grade specifications presented in the supporting dossiers and produced consistent with cGMP are GRAS based on scientific procedures.

It is our opinion that other qualified experts would concur with these conclusions.

(b) (6)

Joseph F. Borzelleca, Ph.D.
Virginia Commonwealth University School of Medicine

27 July 2010
Date

(b) (6)

William J. Waddell, M.D.
University of Louisville School of Medicine

30 July 2010
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APPENDIX B

APPENDIX B

METHODS OF ANALYSIS

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APPENDIX C

APPENDIX C

CERTIFICATES OF ANALYSIS AND COMPOSITIONAL DATA

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SUBMISSION END