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Glico

EZAKI GLICO CO., LTD.
Institute of Health Sciences
4-6-5 Utajima Nishiyodogawa-ku
Osaka 555-8502 Japan
Phone: 81-6-6477-8425
Fax : 81-6-6477-8362

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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 stearothermophilus* TRBE14 (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-D-glucan branching enzyme preparation from *Bacillus stearothermophilus* TRBE14, 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replaces the original Notice for 1,4-α-glucan branching enzyme, which was submitted on October 7, 2010 and subsequently withdrawn on January 31, 2011 (GRN 000361).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-D-glucan branching enzyme preparation from *Bacillus stearothermophilus* TRBE14 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-D-glucan branching enzyme preparation from *Bacillus stearothermophilus* TRBE14, 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-D-glucan branching enzyme preparation from *Bacillus stearothermophilus* TRBE14.

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 stearothermophilus* TRBE14 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 stearothermophilus* TRBE14 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 non-genetically-modified *Bacillus stearothermophilus* TRBE14 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
Chief Scientific Officer
Ezaki Glico Co., Ltd
4-6-5, Utajima, Nishioyogogawa
Osaka, Japan

Telephone: (06)6477-8425
Facsimile: (06)6477-8362
Email: kuriki-takashi@glico.co.jp

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 *B. stearothermophilus* TRBE14 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. The typical use level of the 1,4-α-glucan branching enzyme preparation is 500 U/g substrate in the production of cyclic dextrin, highly branched. The 1,4-α-glucan 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 *B. stearothermophilus* TRBE14 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 branching enzyme 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 1,4-α-glucan branching enzyme preparation from *B. stearothermophilus* TRBE14 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 *B. stearothermophilus* TRBE14, 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.

The 1,4-α-glucan branching enzyme preparation from *B. stearothermophilus* TRBE14 is GRAS based on scientific procedures for its intended use as a processing aid in the production of the modified food starch ingredients, 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 from *B. stearothermophilus* TRBE14, 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-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 has a molecular weight between 62,000 and 66,000 g/mol. The amino acid sequence of the 1,4-α-glucan branching enzyme is as follows:

```
MIAANPTDLEVYLFHEGSLYKSYELFGAHVINEGGKVGTRFCVWAPHAREVRLVGSFNDWDGTDFRLEKV
NDEGVWTIVVPENLEGHLYKYEIVTPDGQVLFKADPYAFYSELRPHTASIAYDLKGYQWNDQSWKRKKRR
KRIYDQPMVIYELHFGSWKKKDGRFYTYREMADELISYVLDHGFTHIELLPLVEHPLDRSWGYQGTGYYA
VTSRYGTPHDFMYFVDRCHQAGIGVIMDWVPGHFCKDAHGLYMFDGAPTYEYANEKDRENYVWGTANFDL
GKPEVRSFLISNALFWLEYYHIDGFRVDAVANMLYWPNNDRLYENPYAVEFLRKLNEAVFAYDPNALMIA
EDSTDWPKVTAPTYEGGLGFNYKWNMGWMNDMLKYMETPPYERRHVHNQVTFSLLYAYSENFILPFSHDE
VVHGKKSLLNKMPGSYEEKFAQLRLLYGYMMAHPGKKLLFMGNEFAQFDEWKFEDELDWVLFDFELHRKM
NDYMKELIACYKRYKPFYELDHDPQGFEWIDVHNAEQSIFSFIRRGKKEDDVLVIVCNFTNQAYDDYKVG
VPLLVPYREVLNSDAVTFGGSGHVNGKRLSAFNEPFHGKPYHVRMTIPPFGISILRPVQKRGERKRNEKE
MHRHVIGRRARKSASLADDKHR
```

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 the formation of glycogen.

The 1,4-α-glucan branching enzyme preparation from *B. stearothermophilus* TRBE14 is thermostable, with optimal activity at a temperature of 50°C and pH 7.5 (Takata *et al.*, 1994). The 1,4-α-glucan branching enzyme preparation 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 the 1,4-α-glucan branching enzyme preparation is 40,000±10,000 U/mL, 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-308, Asp-419, and Glu-351 in the 1,4-α-glucan branching enzyme expressed by *B. stearothermophilus*. 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

The production strain is non-genetically modified *B. stearothermophilus* TRBE14. Isolation and identification of *B. stearothermophilus* TRBE14 has been previously described by Takata *et al.* (1994). Briefly, thermophilic bacteria that produce 1,4-α-glucan branching enzyme were initially isolated based on their ability to accumulate glycogen, as the enzyme is known to be involved in glycogen synthesis. The strains were isolated from a soil sample on peptone-yeast-glucose (PYG) medium at 60°C using an iodine solution[3], and then grown overnight. Cell extracts were prepared from the bacterial cultures, and were first incubated with an amylose solution, and the resultant mixture was then incubated in the presence of α-amylase activity. Extracts exhibiting 1,4-α-glucan branching enzyme activity were those that produced 6^3-*O*-α-glucosyl-maltotriose as the smallest dextrin. A cell extract from a strain designated as TRBE14 was shown to produce the largest amount of 6^3-O-α-glucosyl-maltotriose, and exhibited the greatest or most efficient 1,4-α-glucan branching enzyme activity. This strain was therefore selected for production, and was characterized as a strictly aerobic, Gram-positive, endospore-forming bacillus. The bacterial cell was 0.6 to 0.8 μm in diameter and 2 to 3 μm in length. The optimal temperature range for growth was determined to be 40 to 70°C at pH 7; no bacterial growth occurred at pH 5.7.

[3] Strains that accumulate glycogen have been shown to stain brown in the presence of an iodine solution (Govons *et al.*, 1969).

II.A.4 Composition of the Enzyme Preparation

II.A.4.1 Levels of Diluents, Stabilizers, and Preservatives in the Branching Enzyme Preparation

The 1,4-α-glucan branching enzyme preparation does not contain any diluents or carriers. Small quantities of ethanol and lysozyme (from egg whites) are added to the final enzyme preparation for preservative purposes. Upon application of the 1,4-α-glucan branching enzyme preparation in food processing, the resulting ethanol content in the reaction mixture is marginal at a level of 0.15 to 0.25 % (w/w) (or 1,500 to 2,500 ppm). No residual lysozyme is expected to be present in the final food ingredient (*i.e.,* cyclic dextrin, highly branched) as it is removed in the ion-exchange purification step.

II.A.4.2 Presence of Secondary Enzyme Activities

The 1,4-α-glucan branching enzyme preparation may contain weak α-amylase and α-glucosidase activity. These activities are assessed for every batch of the enzyme preparation for quality control purposes, ensuring that the levels of glucose and reducing sugars are not above those set out in the product specifications for the final ingredient. The activities are assessed using the glucose-oxidase/peroxidase method and the results presented are relative to the validated control batch (b) (4) [redacted], since the absolute values obtained are variable. A relative value of ≥2 is considered to be a positive result. The results of analyses of 3 non-consecutive batches of the enzyme preparation are summarized in Table II.A.4.2-1, and demonstrate that the relative levels of glucose and reducing sugars produced are consistently less than 2. A description of the method of analysis and a summary of the analytical results are provided in Appendix B and C, respectively.

Table II.A.4.2-1 Analysis of α-Amylase and α-Glucosidase Activity for Manufactured Batches of the 1,4-α-Glucan Branching Enzyme Preparation

Manufacturing Batch Number	Relative Values (Test Batch/Control Batch[a])	
	Glucose	Reducing Sugars
(b) (4) [redacted]	0.71	0.77
	1.84	1.71
	0.61	0.61
	0.65	0.72
	1.25	1.21
	0.75	0.63
	0.59	0.89

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 were analyzed for protein plus nucleic acid, carbohydrate, fat, water, ash, and total organic solids (TOS) content. The ash and water content ranged from <0.1 to 2.4% and 89.9 to 93.2%, respectively. Protein content was determined by the Kjeldahl method, and ranged from 2.2 to 8.4%. The Kjeldahl method is a quantitative measure of the total nitrogen content in organic compounds, and 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 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 9.5 to 9.7%. In addition, the fat content was determined to be <0.1%. Reports of analysis are provided in Appendix C.

II.B Method of Manufacture

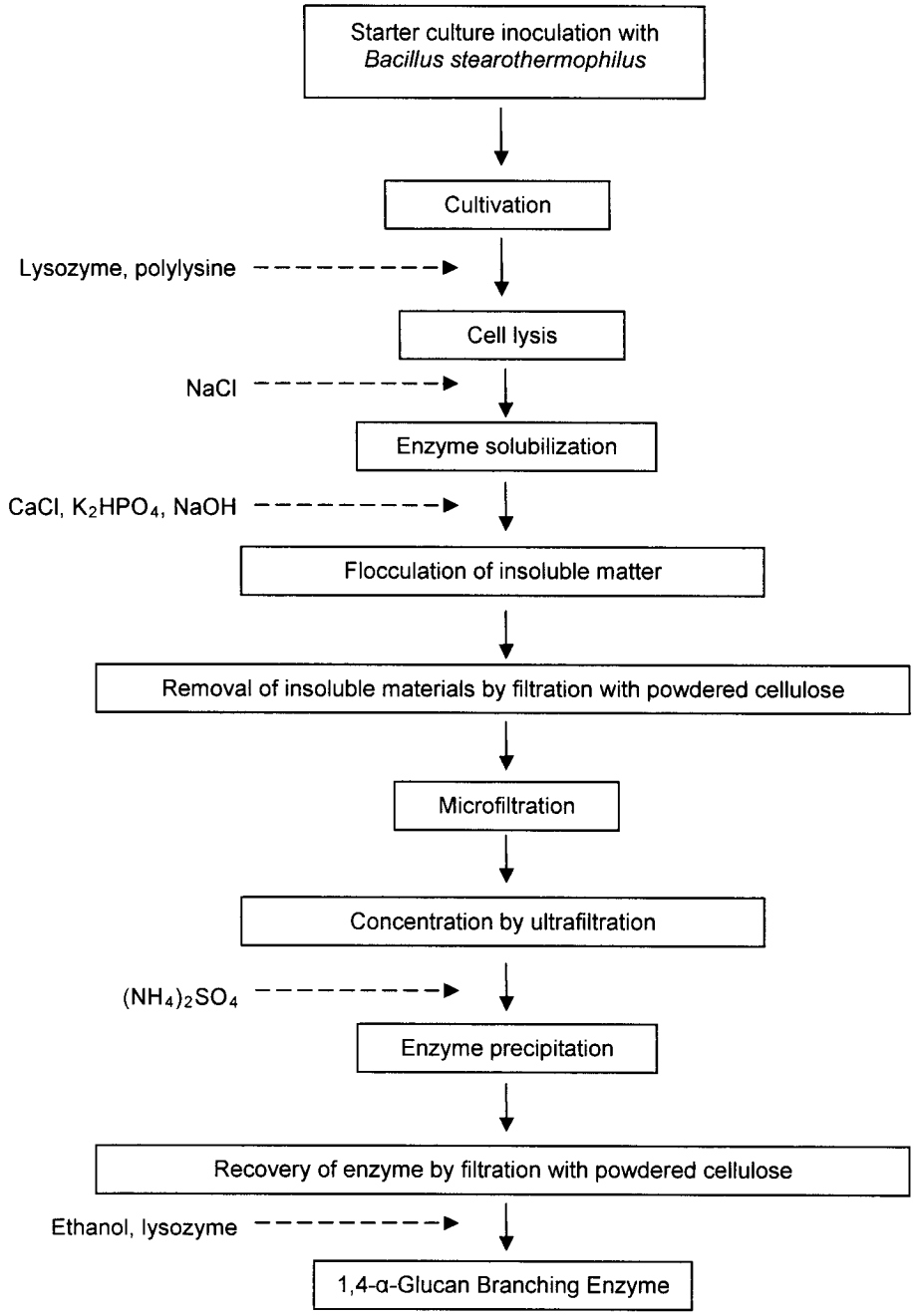
The bacterial production strain is cultivated following the basic manufacturing steps illustrated in Figure II.B-1. The 1,4-α-glucan branching enzyme preparation is manufactured in accordance with cGMP using food-grade materials and using a quality-controlled fermentation and purification process. First, 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. 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 manufacturing 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. Following cultivation of the production bacteria, the bacteria are lysed using lysozyme with ε-polylysine at neutral pH to retrieve the intracellular branching enzyme. The bacteria are then flocculated with the aid of a flocculating agent, monobasic calcium

phosphate (formed from calcium chloride and K_2HPO_4). The insoluble materials are removed from the branching enzyme solution by filtration with diatomaceous earth or powdered cellulose (ε-polylysine is expected to be removed with insoluble materials at this step, and any remaining residues would be removed in the subsequent ultrafiltration step). The solution is further purified by microfiltration and is concentrated by ultrafiltration. The enzyme solution is then concentrated through precipitation of the enzyme with ammonium sulfate, and the enzyme is recovered by filtration with powdered cellulose in order to obtain sufficient enzyme activity per unit. Removal of the bacterial remnants is carried out at the filtration steps, preventing carry-over of the bacteria into the final enzyme preparation. Ethanol and lysozyme are added to the 1,4-α-glucan branching enzyme preparation for preservative purposes. Water, ethanol, and lysozyme also are added to adjust the enzymatic activity of the 1,4-α-glucan 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 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 antimicrobial activity. The reason for this non-inclusion is that the 1,4-α-glucan branching enzyme preparation contains ingredients that may act as antimicrobial agents (*i.e.,* ethanol and lysozyme), which 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 antimicrobial activity. The product specifications include those for the physical appearance and activity of the 1,4-α-glucan 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)	40,000±10,000 U/mL	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]

As_2O_3 = Arsenic oxide; AAS = atomic absorption spectrophotometry; BGLB = brilliant green lactose bile; CFU = colony forming unit; FCC = Food Chemical Codex
[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/25 g.

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 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) 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) and thus, consists of a cyclization reaction resultant of intramolecular transglycosylation, forming highly branched cyclic dextrins.

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 1,4-α-glucan branching enzyme preparation 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 in the production of modified food starch ingredients, the enzyme preparation itself is not added directly to food. Moreover, the manufacturing processes for the cyclic dextrin, highly branched ingredient incorporate steps to ensure inactivation and removal of the 1,4-α-glucan branching enzyme preparation. The 1,4-α-glucan 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. Thus, carry-over of the 1,4-α-glucan branching enzyme preparation into the final food ingredient, or the final food product, is negligible to none. This is supported by analyses of 3 non-consecutive lots of each of the cyclic dextrin, highly-branched ingredient, which indicated 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%).

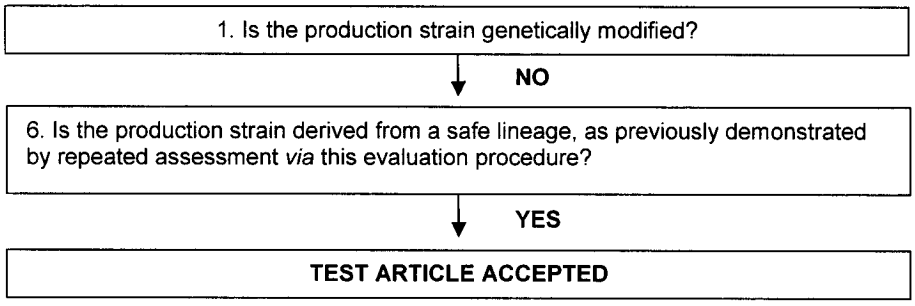
IV. SAFETY OF 1,4-α-GLUCAN BRANCHING ENZYME PREPARATION

IV.A Safety of the Production Microorganism

Native *B. stearothermophilus* strain TRBE14 is used as the production strain for the 1,4-α-glucan branching enzyme preparation. *B. stearothermophilus* is a non-toxigenic and non-pathogenic Gram-positive bacterium, and is used in food processing as a safe source of native enzymes (Pariza and Johnson, 2001; Olempska-Beer *et al.*, 2006). The 1,4-α-glucan branching enzyme from *B. stearothermophilus* has no 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.

The safety of the production strain was determined using the guidelines developed by Pariza and Foster (1983), Pariza and Johnson (2001), and the International Food Biotechnology Council (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 approach, 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 *Bacillus stearothermophilus* for Use in Food



Modified from Pariza and Johnson (2001).

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

IV.B.1 Genotoxicity Studies

The potential genotoxicity of the 1,4-α-glucan branching enzyme preparation was assessed in the L5178Y $TK^{+/-}$ mouse lymphoma forward mutation assay (Choi *et al.*, 2009). This study was conducted in accordance with the Ministry of Health and Welfare (MHW) Guidelines for Toxicity Studies of Drugs and the Organization for Economic Cooperation and Development (OECD) Guideline for the Testing of Chemicals Test No. 476, as well as in compliance with the MHW and OECD principles of Good Laboratory Practice (GLP) (OECD, 1997, 1998). Assays were conducted in the presence and absence of rat liver microsomal S9 metabolic activation. The culture solution vehicle served as a negative control. Positive controls included methyl methanesulfonate for assays conducted in the absence of S9 metabolic activation and cyclophosphamide for assays conducted in the presence of S9 metabolic activation. Cells were treated with the branching enzyme preparation at concentrations of 156, 313, 625, 1,250, 2,500, or 5,000 µg/mL. The branching enzyme preparation was not cytotoxic at any concentration either with or without S9 metabolic activation. No significant difference in the induction of mutation frequency was observed between the branching enzyme preparation and the negative control either in the presence or absence of S9 metabolic activation. Positive controls significantly increased the frequency of mutations compared to the negative control, confirming the sensitivity of the test. Based on these findings, the 1,4-α-glucan branching enzyme preparation is non-genotoxic as assessed in the mouse lymphoma assay.

IV.B.2 Acute Toxicity Studies

The acute toxicity of the 1,4-α-glucan branching enzyme preparation was examined in a group of 5 male and 5 female SLC-Wistar rats that were weighed and then administered a single dose of 2,000 mg/kg body weight of the 1,4-α-glucan branching enzyme preparation by oral gavage following a 16-hour fast (Choi *et al.*, 2009). This study was conducted in compliance with the GLP Standard Ordinance for Non-Clinical Laboratory Studies on Safety of Drugs, and in accordance with the Revision of the Guidelines for Single-Dose and Repeated-Dose Toxicity Studies established by the MHW. The animals were fasted for a further 3 hours following administration of the branching enzyme preparation. For the 6 hours immediately following administration of the branching enzyme preparation, the rats were observed at 1-hour intervals and then every day thereafter for a period of 14 days. On weekdays, the animals were observed twice daily and on weekend days, they were observed once daily. Viability and signs of toxicity were assessed and documented. The body weight of the animals was recorded on Days 7 and 14 of the observation period. All animals were necropsied on Day 14 of the observation period and organs examined macroscopically upon necropsy. None of the animals died during the 14-day observation period following the administration of the branching enzyme preparation and no abnormalities were observed in the general condition of the animals at any

time point during the 14-day study. All rats displayed satisfactory body weight changes throughout the observation period. No compound-related macroscopic abnormalities were detected upon necropsy in any of the animals. Based on the 100% survival rate during the 14 days following oral administration of 2,000 mg/kg body weight of the branching enzyme preparation, the oral LD_{50} was considered to be greater than 2,000 mg/kg body weight. The 1,4-α-glucan branching enzyme preparation was therefore considered to have a low order of toxicity following acute oral exposure.

IV.C Additional Safety Considerations

IV.C.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 process outlined in Section II.B. Moreover, the manufacturing process for the final 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 ingredient demonstrated absence of any 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 commercial cyclic dextrin, highly branched in Japan.

IV.D 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 1,4-α-glucan branching enzyme preparation:

1) All of the processed foods and beverages containing the modified food starch ingredients (*i.e.,* cyclic dextrin, highly branched) were produced using the branching enzyme preparation as a processing aid;
2) All TOS in the 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 365 U/mg TOS (the average of 3 batches), and is being used as a processing aid in the production of processed foods and beverages containing 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/365 U = 1.36 mg TOS/g substrate

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. Based on the assumption that the entire enzyme 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 would be:

1.36 mg TOS/g x 1,011 mg/kg body weight/day = 1.37 mg TOS/kg body weight/day

The oral LD_{50} of the 1,4-α-glucan branching enzyme preparation (enzymatic activity of 34,000 U/g) was determined to be greater than 2,000 mg/kg body weight in an acute oral toxicity study (see Section IV.B.2). Considering the enzymatic activity and an average value of 365 U/mg TOS, the oral LD_{50} of the 1,4-α-glucan branching enzyme preparation corresponds to 68,000 U/kg body weight (2,000 mg/kg body weight x 34,000 U/g) or 186.3 mg TOS/kg body weight. On a daily basis, this is approximately 136 times greater than the amount of enzyme that would be expected to be consumed by humans under the "worst-case" scenario.

V. SUMMARY AND BASIS FOR GRAS CONCLUSION

The GRAS determination for the use of 1,4-α-glucan branching enzyme preparation from *B. stearothermophilus* TRBE14 as a processing aid in food production is based on scientific procedures. 1,4-α-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] is produced from non-genetically-modified *B. stearothermophilus* TRBE14. The 1,4-α-glucan 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 has also been compositionally characterized and analyzed for other enzymatic activities. The 1,4-α-glucan branching enzyme preparation was identified as harboring weak α-amylase and α-glucosidase activities. These activities are assessed for every lot of the 1,4-α-glucan branching enzyme preparation and are under strict control.

The 1,4-α-Glucan branching enzyme 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 . The typical use level of the 1,4-α-glucan branching enzyme preparation is 500 U/g substrate in the production of cyclic dextrin, highly branched, which is intended for use in a variety of traditional foods and beverages. As the 1,4-α-glucan branching enzyme preparation is used only as a processing aid, it will not be added directly to food and the manufacturing process for the final food ingredient (*i.e.*, cyclic dextrin, highly branched) incorporates steps to ensure inactivation and removal of the 1,4-α-glucan 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.

The safety of the 1,4-α-glucan branching enzyme preparation was supported by the safety guidelines for microbial enzyme preparations developed by Pariza and Foster (1983), Pariza and Johnson (2001), and IFBC (1990), as well as the fact that the *B. stearothermophilus* strain TRBE14 is considered to be a non-toxigenic and non-pathogenic Gram-positive bacteria. Based on the safety of the production strain, 1,4-α-glucan branching enzyme preparation is considered to be safe for use in food production.

The safety of 1,4-α-glucan branching enzyme preparation was further assessed in standard toxicological tests, specifically in an acute oral toxicity study and in an *in vitro* genotoxicity

assay. The 1,4-α-glucan branching enzyme preparation was not acutely toxic in rats; the acute oral LD_{50} in Sprague-Dawley rats was greater than 2,000 mg/kg body weight (Choi *et al.*, 2009). Furthermore, the 1,4-α-glucan branching enzyme preparation was non-genotoxic in a mouse lymphoma assay conducted in L5178Y $TK^{+/-}$ cells at concentrations of up to 5,000 µg/mL (Choi *et al.*, 2009). Collectively, the results of these studies indicate that the use of 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 1,4-α-glucan branching enzyme preparation from *B. stearothermophilus* TRBE14 for use in the production of cyclic dextrin, highly branched, produced consistently with cGMP and meeting appropriate food grade specifications described herein, are safe and suitable.

The Expert Panel likewise unanimously concluded that the intended uses of 1,4-α-glucan branching enzyme preparation from *B. stearothermophilus* TRBE14 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 from non-genetically-modified *B. stearothermophilus* TRBE14 is GRAS based on scientific procedures for its intended use as a processing aid in the production of the modified food starch ingredients, such as 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
Date

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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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