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SENT *VIA* FEDEX

October 8, 2010

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: GRAS Notice for 1,4-α-D-Glucan Branching Enzyme

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, 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*. 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 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, 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.

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.

Sincerely,

(b) (6)

Takashi Kuriki
Chief Scientific Officer

Encl.

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GRAS Exemption Claim for 1,4-α-D-Glucan Branching Enzyme 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

October 7, 2010

GRAS Exemption Claim for 1,4-α-D-Glucan Branching Enzyme 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-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) has been determined to be Generally Recognized as Safe (GRAS) 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 branching enzyme in food production as described below is exempt from the requirement of premarket approval.

Signed,

(b) (6)

October 19, 2010
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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4-6-5, Utajima, Nishioyogogawa
Osaka, Japan

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I.C Common Name of the Notified Substance

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

I.D Conditions of Intended Use in Food

The branching enzyme is intended for use as a processing aid in food production. Specifically, the branching enzyme 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 and enzymatically-synthesized glycogen[2] produced from food-grade dextrin. The typical use level of the branching enzyme is 500 U/g substrate in the production of cyclic dextrin, highly branched and 5000 U/g substrate in the production of enzymatically-synthesized glycogen. The branching enzyme 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, 2010), 1,4-α-D-glucan branching enzyme has been determined to be GRAS based on scientific procedures for use in the production of cyclic dextrin, highly branched and enzymatically-synthesized glycogen.

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 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 Glico Co., Ltd. (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.

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[1] A GRAS Exemption Claim for cyclic dextrin, highly branched [trade name Cluster Dextrin® (CCD®)] has been concurrently submitted to FDA.
[2] A GRAS Exemption Claim for enzymatically-synthesized glycogen will be submitted to FDA in the near future.

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[3] (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-D-glucan branching enzyme, 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-D-Glucan branching enzyme 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 and enzymatically-synthesized glycogen; 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.

[3] 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 branching enzyme 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 Source and Identity

The branching enzyme may be produced *via* one of three production methods. In this regard, the branching enzyme is produced using non-genetically modified *B. stearothermophilus* TRBE14 or *B. subtilis* 168 genetically modified to express the 1,4-α-D-glucan branching enzyme structural gene from either *B. stearothermophilus* TRBE14 or *Aquifex aeolicus* VF5. The gene that encodes the 1,4-α-D-glucan branching enzyme is *glgB*. 1,4-α-D-Glucan branching enzyme is the resultant protein product of all sources and performs the same catalytic activity (described in detail in Section IV.B) irrespective of the production strain. Thus, the branching enzyme subject of this GRAS exemption claim is the same irrespective of the production method.

The branching enzyme preparation is a dark-brown solution. The enzyme activity of the branching enzyme is >10,000 U/mL and is highly specific to branching enzyme activity. Specifically, this activity consists of catalyzing an intra- or intermolecular transglycosylation reaction as described in Section IV.B. The catalytic residues consist of 2 aspartic acid (Asp) residues and 1 glutamic acid (Glu) residue. These residues are conserved among *B. stearothermophilus* TRBE14 and *A. aeolicus* VF5. The catalytic residues have also been identified as important for substrate-binding. .

Common or Usual Name:	1,4-α-D-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
Molecular weight:	Between 62,000 and 66,000 g/mol

II.B Method of Manufacture

The branching enzyme may be produced *via* 1 of 3 production methods. In the first, the branching enzyme is derived from non-genetically modified *B. stearothermophilus* TRBE14. In the second and third methods, the branching enzyme is produced using *B. subtilis* strain 168 genetically modified to express the branching enzyme from either *B. stearothermophilus* TRBE14 or *A. aeolicus* VF5. For purposes of identifying the production strain/method, the branching enzyme when derived from the non-genetically modified production strain is referred to as BE and those derived from the genetically modified production strain are referred to as BE-01 and BE-02, respectively. However, the resultant protein product is 1,4-α-D-glucan branching enzyme irrespective of the production strain/method used. The genetically modified production strains are produced using standard genetic modification techniques and the branching enzyme is manufactured in accordance with cGMP using food-grade materials.

II.B.1 Construction of Genetically Modified Microorganisms

The host microorganism subject to genetic modification is *B. subtilis* 168. To produce *B. subtilis* 168 genetically modified to express the branching enzyme from *B. stearothermophilus* TRBE14, the *glgB* gene encoding for 1,4-α-D-glucan branching enzyme was cloned from the genomic DNA of the donor microorganism, *B. stearothermophilus* TRBE14, as described by Takata *et al.* (1994). A *Bam*HI restriction site was inserted upstream of the transcriptional start site of the *glgB* gene and an *Eco*RI site was inserted downstream of the stop codon. The resultant DNA fragment was inserted between *Bam*HI and *Eco*RI restriction sites of vector pUB110 to construct the plasmid pUBE183. The pUBE183 plasmid was then transfected into *B. subtilis* 168; the plasmid is not integrated into the genome of *B. subtilis* 168. The branching enzyme when produced from this production strain is referred to as BE-01 for practical identification purposes.

Production of *B. subtilis* 168 genetically modified to express the branching enzyme from *A. aeolicus* VF5 involved chemically synthesizing the *glgB* gene based on the amino acid sequence of 1,4-α-D-glucan branching enzyme as expressed by *A. aeolicus* strain VF5 (Takata *et al.*, 2003). The *glgB* gene was synthesized with a *Bam*HI site (underlined) and a ribosome binding 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:

> CTT<u>GGATCC</u>TGATTAAA<u>AGGAGGT</u>AATAACAT*ATG*...(coding sequences)...*TAA*T<u>GAATTC</u>TTC

The synthetic DNA fragment was digested with *Bam*HI and *Eco*RI restriction enzymes and then inserted between the *Bam*HI and *Eco*RI restriction sites of vector pUB110 to construct the plasmid pUAQ2. The pUAQ2 plasmid was then transfected into *B. subtilis* 168, without

integration into genomic DNA. The branching enzyme when produced from this production strain is referred to as BE-02.

II.B.2 Fermentation

All production bacterial strains are cultivated following the same basic manufacturing steps, as illustrated below in Figure II.B.2-1. The 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 strains 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. The culture medium also contains corn steep liquor, which provides the bacteria with a source of organic nitrogen (amino acids), as well as vitamins and other nutrients, to support growth. Dextrose, sucrose, sorbitol, or glycerol are 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 ($MnCl_2 \cdot 4H_2O$) as a source of manganese (ii) 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 to produce 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 federal regulations as indicated in Table II.B.2-1.

The main liquid culture medium is then 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 or without ε-polylysine at neutral pH. In the production of BE-02, the culture is heat-treated prior to cell lysis. The heat treatment serves to effectively denature any undesirable proteins. Following heat treatment, the bacteria are flocculated with the aid of a flocculating agent, monobasic calcium phosphate (formed from calcium chloride and K_2HPO_4), and then recovered by filtration with diatomaceous earth. After the cell lysis step, the branching enzyme is solubilized and insoluble materials flocculated with the aid of a flocculating agent, monobasic calcium phosphate which slowly forms from calcium chloride and K_2HPO_4, allowing for the flocculation of insoluble materials. This flocculation step is not incorporated into the manufacturing process for BE-02, since the pH of the enzyme solution is high as a result of the addition of sodium carbonate in the enzyme solubilization step. Monobasic calcium phosphate

does not form at alkaline pH, and therefore, flocculation is effective only at neutral pH. Omission of this step, however, does not affect the yield or the purity of BE-02 as adequate upstream and downstream purification steps are included to remove any insoluble impurities (*i.e.*, heat treatment and filtration with diatomaceous earth). 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, but also would be removed in the subsequent the ultrafiltration step). The solution is then further purified by microfiltration and are concentrated by ultrafiltration. In the production of BE the enzyme solution is further concentrated through precipitation of the enzyme with ammonium sulfate and recovery of the enzyme 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, as well as lysozyme for BE, is added to BE and BE-01 for additional preservative purposes, which is not required for BE-02. No other diluents, carriers, or stabilizers 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 quality and consistent product. Chemical and microbiological analyses are performed on the final enzyme preparation to ensure absence of heavy metal and microbiological contamination as outlined in Section II.C.

Table II.B.2-1 List of Processing Aids Used in the Manufacture of 1,4-α-D-Glucan Branching Enzyme

Processing Aid	Function	Reference to Appropriate Use in Food
Protease derived from *Bacillus licheniformis*	Hydrolysis of soy bean powder	21 CFR §184.1027 Mixed carbohydrase and protease enzyme preparation (U.S. FDA, 2010) 21 CFR §184.1150 Bacterially-derived protease enzyme preparation (U.S. FDA, 2010)
Peptones from enzyme-hydrolyzed casein and soy bean powder (produced from defatted soy beans)	Component of culture medium	21 CFR §184.1553 Peptones (U.S. FDA, 2010)
Yeast extract derived from *Saccharomyces cerevisiae*	Component of culture medium	21 CFR §184.1983 Bakers yeast extract (U.S. FDA, 2010)
Corn steep liquor	Component of culture medium	21 CFR §73.275 Dried algae meal (U.S. FDA, 2010) No specific regulations pertaining to corn steep liquor; however, corn steep liquor is permitted for use as a media component for the culturing of *Moniliella pollinis* and *Lactococcus lactis*, which are used in the production of erythritol and nisin, respectively (GRAS Notice Nos. GRN 000076 and 000065, U.S. FDA, 2001a,b)
Dextrose	Component of culture medium	21 CFR §184.1857 Corn sugar (U.S. FDA, 2010)
Sucrose	Component of culture medium	21 CFR §184.1854 Sucrose (U.S. FDA, 2010)

Table II.B.2-1 List of Processing Aids Used in the Manufacture of 1,4-α-D-Glucan Branching Enzyme

Processing Aid	Function	Reference to Appropriate Use in Food
Sorbitol	Component of culture medium	21 CFR §184.1835 Sorbitol (U.S. FDA, 2010)
Glycerol	Component of culture medium	21 CFR §182.1320 Glycerin (U.S. FDA, 2010)
Manganese (II) chloride tetrahydrate	Component of culture medium	21 CFR §184.1446 Manganese chloride (U.S. FDA, 2010)
Sodium chloride	Component of culture medium and processing aid in solubilization of BE	21 CFR §182.1 Substances that are generally recognized as safe (U.S. FDA, 2010)
Potassium dihydrogen phosphate and dipotassium hydrogen phosphate	Component of culture medium	21 CFR §182.6285 Sequestrants (dipotassium phosphate) (U.S. FDA, 2010) No specific regulations pertaining to potassium dihydrogen phosphate; however, many potassium and phosphate salts are GRAS as direct food substances
Sodium hydroxide	pH control agent	21 CFR §184.1763 Sodium hydroxide (U.S. FDA, 2010)
30% Food-grade silicone (dimethylpolysiloxane) emulsion	Anti-foaming agent	21 CFR §173.340 Defoaming agents (U.S. FDA, 2010)
Lysozyme	Cell lysis aid and preservative	No specific regulation pertaining to lysozyme; however, lysozyme is permitted for use as an antimicrobial agent on casings for frankfurters and on cooked meat and poultry products as an antimicrobial agent (GRAS Notice No. GRN 000064, U.S. FDA, 2001c)
ε-Polylysine	Cell lysis aid	No specific regulation pertaining to ε-polylysine; however, polylysine is permitted for use as an antimicrobial agent in cooked rice and sushi rice (GRAS Notice No. GRN 000135, U.S. FDA, 2004)
Calcium chloride	Flocculating agent	21 CFR §184.1193 Calcium chloride (U.S. FDA, 2010)
Ammonium sulfate	Solubilization of BE-01 and precipitation of BE	21 CFR §184.1143 Ammonium sulfate (U.S. FDA, 2010)
Sodium carbonate	Solubilization of BE-02	21 CFR §184.1742 Sodium carbonate(U.S. FDA, 2010)
Diatomaceous earth	Filter medium	21 CFR §178.3297 Diatomaceous earth (U.S. FDA, 2010)
Powdered cellulose	Filtration aid	21 CFR §177.2260 Filters, resin-bonded (U.S. FDA, 2010)
Filter consisting of a polyethylene membrane with a polysulfone housing module	Microfiltration	21 CFR §177.1600 Polyethylene resins, carboxyl modified (U.S. FDA, 2010) 21 CFR §177.1655 Polysulfone resins (U.S. FDA, 2010)
Filter consisting of a polyvinylidene fluoride membrane with a polysulfone housing module	Microfiltration	21 CFR §177.2510 Polyvinylidene fluoride resins (U.S. FDA, 2010)

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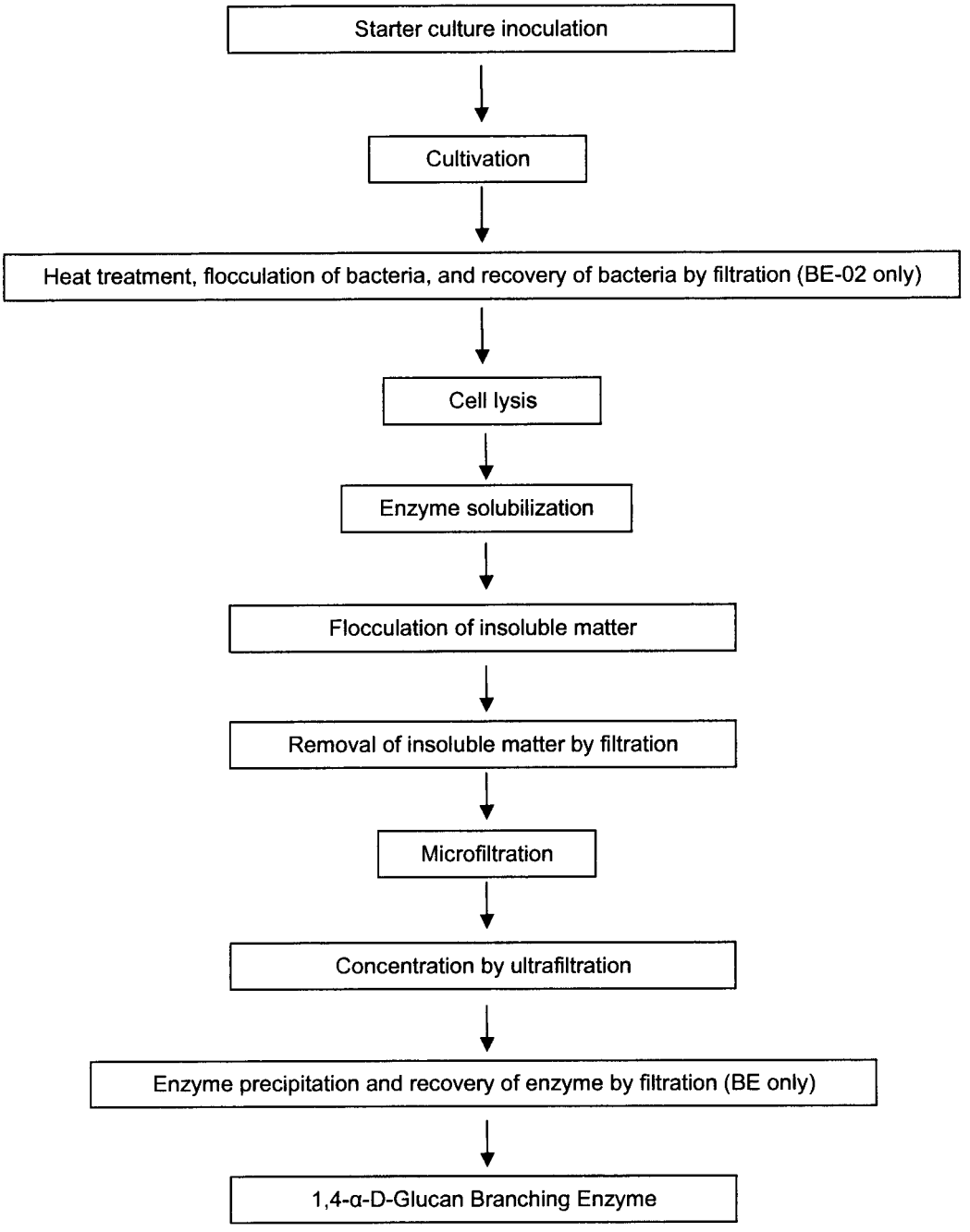
Table II.B.2-1 List of Processing Aids Used in the Manufacture of 1,4-α-D-Glucan Branching Enzyme

Processing Aid	Function	Reference to Appropriate Use in Food
Ultrafiltration module consisting of a polyacrylonitrile membrane and polysulfone housing module	Microfiltration	21 CFR §177.2910 Ultra-filtration membranes (U.S. FDA, 2010)
Ethanol	Preservative	21 CFR §184.1293 Ethyl alcohol (U.S. FDA, 2010) 21 CFR §172.340 Fish protein isolate (U.S. FDA, 2010)

Figure II.B.2-1 Schematic Overview of the Manufacturing Process for 1,4-α-D-Glucan Branching Enzyme



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II.C Specifications and Analytical Data

The product specifications for the branching enzyme preparation are presented in Table II.C-1. The specifications for the branching enzyme comply with current specifications for enzyme preparations of the Joint FAO/WHO Committee on Food Additives (JECFA) (JECFA, 2006), as well as the Food Chemicals Codex (FCC) (FCC, 2008). 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.

Analyses of 3 non-consecutive lots of the branching enzyme preparation produced from each of the three production methods confirm that the material produced by the manufacturing process is consistent and complies with the product specifications. The analytical data demonstrate the absence of any chemical impurities or microbial contamination. The complete certificates of analysis for these lots are provided in Appendix C.

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

Specification Parameter	Specification Requirements	Method of Analysis
Appearance	Dark brownish liquid	Visual examination
Assay (Activity)[1]	Greater than 10,000 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)
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 (not applicable for BE)	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; BE = 1,4-α-D-glucan branching enzyme preparation produced using *Bacillus stearothermophilus* TRBE14; 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. 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 obtain will not be used in food production. See Appendix B for a description of the method of analysis.

II.D Additional Compositional Characterization

II.D.1 Protein, Nucleic Acid, Carbohydrate, Fat, Water, Ash, and Total Organic Solids Analyses

Three (3) non-consecutive lots of the branching enzyme preparation produced from each of the three production methods 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 therefore, the values represent protein plus nucleic acid content. The protein content of the branching enzyme preparation ranged from 2.2 to 8.4%. 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. The TOS content of the branching enzyme preparation ranged from 4.4 to 9.7%. In addition, the fat content was determined to be <0.1%. Reports of analysis are provided in Appendix C.

Exclusive in these analyses are analytical data for the major known milk allergen, casein, which is used as a component of the culture medium; however, the final cyclic dextrin, highly branched and enzymatically-synthesized ingredients are free of any protein content (limit of detection of 0.1% for total protein), indicating that casein is not present in the final ingredients that are added to food. Removal of such constituents, and of the branching enzyme, is resultant of the purification process applied to the final ingredients. In support of the absence of allergenic levels of casein in the final product, no adverse events, including those of an allergenic nature, have been reported from the consumption of a commercial cyclic dextrin, highly-branched-based sports drink in Japan. Furthermore, no other companies in Japan that use cyclic dextrin, highly branched have reported adverse events from its use.

III. SELF-LIMITING LEVELS OF USE

The branching enzyme is used only at levels necessary to achieve the intended effect. Production costs prevent the application of excess amounts of the branching enzyme. There are no self-limiting levels of use pertaining to the quality of the food ingredients produced.

IV. BASIS FOR GRAS DETERMINATION

IV.A Documentation to Support the Safety of 1,4-α-D-Glucan Branching Enzyme

The determination that 1,4-α-D-glucan branching enzyme is GRAS is on the basis of scientific procedures, and the information supporting the general recognition of the safe includes:

- data pertaining to the identity and intended uses of the branching enzyme;
- data pertaining to the safety of the microorganisms used as the production source of the branching enzyme; and
- preclinical toxicity studies conducted with the final branching enzyme preparation produced using all three methods.

Moreover, these data were reviewed by a panel of experts, qualified by scientific training and experience to evaluate the safety of ingredients as components of food, who concluded that the intended uses of the branching enzyme as a processing aid in food production are safe and suitable and would be GRAS based on scientific procedures [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"]. A summary of these data is presented herein.

IV.B Technological Function of 1,4-α-D-Glucan Branching Enzyme

The branching enzyme 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 and enzymatically-synthesized glycogen produced from food-grade dextrin. The enzyme activity of the branching enzyme consists of catalyzing 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). The reaction, therefore, consists of a cyclization reaction resultant of intramolecular transglycosylation, forming highly branched cyclic dextrins. A similar reaction also occurs with amylose as the substrate in which intermolecular transglycosylation results in the formation of enzymatically-synthesized glycogen. Specifically, the branching enzyme catalyzes glycogen synthesis from short-chain amylose by transferring the terminal reducing glucose unit from the α-(1,4) glucose chain to an acceptor glucose unit of another amylose chain *via* an α-1,6 glucosidic bond.

IV.C Intended Use of 1,4-α-D-Glucan Branching Enzyme

The branching enzyme is intended for use as a processing aid in food production. As mentioned in Section IV.B, the branching enzyme is specifically intended for use in the production of modified food starch ingredients, including cyclic dextrin, highly branched and enzymatically-synthesized glycogen. The typical use level of the branching enzyme is 500 U/g substrate in the production of cyclic dextrin, highly branched and 5,000 U/g substrate in the production of enzymatically-synthesized glycogen. The branching enzyme is used only at levels necessary to achieve the intended effect.

The cyclin 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. The enzymatically-synthesized glycogen ingredient is intended for use in selected food categories, including ready-to-eat cereals, instant and hot cereals, milk and non-milk based meal replacements, sports, energy, and isotonic drinks, vitamin and enhanced waters, meal replacement bars, yogurt, chocolate, and non-chocolate soft candy. As the branching enzyme is used only as a processing aid, the enzyme itself is not added directly to food.

Moreover, the manufacturing processes for the cyclic dextrin, highly branched and enzymatically-synthesized glycogen ingredients incorporate steps to ensure inactivation and removal of the branching enzyme. The branching enzyme is denatured and inactivated by pH adjustment with hydrochloric acid and by high temperature heating, and purification of the final ingredient by filtration through diatomaceous earth, perlite, and an ion-exchange resin ensures removal of the production enzyme. Thus, carry-over of the branching enzyme into the final food ingredient, and thus into the final food product, is negligible. In support of the negligible carry-over, 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%).

IV.D Safety Evaluation of 1,4-α-D-Glucan Branching Enzyme

IV.D.1 Safety of the Production Strain

Guidelines for evaluating the safety of microbial enzyme preparations used in foods have been established by Pariza and Foster (1983). The International Food Biotechnology Council (IFBC) (1990) further extended the early guidelines of Pariza and Foster (1983) to include steps to evaluate the safety of microbial enzyme preparations developed with the use of genetic modification technologies. Pariza and Johnson (2001) also amended the guidelines developed by Pariza and Foster (1983) to include comparable safety guidelines to those of IFBC for

genetically-modified microorganisms and microbial enzyme preparations derived thereof. The guidelines developed by Pariza and Foster (1983), Pariza and Johnson (2001), and IFBC (1990), presented in the form of a decision tree, are widely accepted by the scientific community and regulatory agencies as criteria for assessing the safety of microbial enzyme preparations used in foods. When assessing the safety of microbial enzyme preparations, Pariza and Foster (1983) and Pariza and Johnson (2001) concluded that the primary consideration should be the safety of the production strain, with particular emphasis on the toxigenic potential of the production strain, specifically, the possible production of toxins that are active *via* the oral route. The pathogenic potential of the production strain also should be considered. Moreover, a safety evaluation of a production strain derived *via* genetic modification is to consider the following elements: the host organism; the donor organism; the DNA to be introduced, including the DNA insert (*i.e.*, the gene of interest) and the vector; and the techniques used (IFBC, 1990; Pariza and Johnson, 2001). These are considered in order to establish a safe strain lineage (Pariza and Johnson, 2001). The safety of 1,4-α-D-glucan branching enzyme is evaluated below using the guidelines and decision tree developed by Pariza and Johnson (2001) and taking into consideration all elements.

IV.D.1.1 Safety of the Host Microorganisms

In the first of the 3 possible production methods, native *B. stearothermophilus* strain TRBE14 is used as the production strain from which the branching enzyme is produced (the branching enzyme is referred to as BE in this instance). *B. stearothermophilus* is a non-toxigenic and non-pathogenic Gram-positive bacterium used in food processing as a safe source of native enzymes (Pariza and Johnson, 2001; Olempska-Beer *et al.*, 2006). Thus, *B. stearothermophilus* is considered safe for use as a production strain used in food processing/production.

In the second and third production methods, the branching enzyme (referred to as BE-01 or BE-02) is produced using genetically modified *B. subtilis* strain 168 as the host organism. *B. subtilis* is a ubiquitous non-pathogenic Gram-positive bacterium that is widely used in food processing as a safe source of native enzymes (de Boer and Diderichsen, 1991; Olempska-Beer *et al.*, 2006). Importantly, *B. subtilis* does not harbor toxigenic potential and does not possess genes that code for *B. cereus*-like toxins (U.S. EPA, 1997; Pedersen *et al.*, 2002). In addition, the wild type *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 is considered safe for use as a production strain and a host microorganism used in food processing, and therefore, may be utilized to derive safe strain lineages by genetic modification techniques.

IV.D.1.2 Safety of the Donor Organisms and the DNA Introduced into Bacillus subtilis 168

Thoroughly characterized microbial strains that are considered safe may be genetically modified to incorporate genes of interest from other strains provided that all new DNA introduced are determined to be safe (IFBC, 1990; Pariza and Johnson, 2001). Despite the history of safe use of *B. subtilis* 168 in foods and the lack of toxigenic and pathogenic potential associated with *B. subtilis* 168, an assessment of the safety of the genetically modified strains derived from *B. subtilis* 168 is necessary. The safety of the donor organisms and, as per Pariza and Johnson (2001), the safety of the DNA introduced into *B. subtilis* 168 were evaluated in order to assess the safety of the genetically modified organisms.

As discussed in Section IV.D.1.1, *B. stearothermophilus* is a non-toxigenic and non-pathogenic Gram-positive bacterium used in food processing as a safe source of native enzymes (Pariza and Johnson, 2001; Olempska-Beer *et al.*, 2006). Since the transfer of a gene from a non-toxigenic and non-pathogenic source to a similarly safe host organism is considered safe for enzyme or ingredient production (IFBC, 1990), the transfer of the *glgB* gene from *B. stearothermophilus* TRBE14 is considered safe for transfer into *B. subtilis* for the purposes of producing 1,4-α-D-glucan branching enzyme. *A. aeolicus* strain VF5 is a Gram-negative bacterium, and a search of the scientific literature has not revealed evidence that this bacterium harbors pathogenic or toxigenic potential. Moreover, the DNA encoding the branching enzyme (the *glgB* gene) from either *B. stearothermophilus* TRBE14 or *A. aeolicus* VF5 do not encode for a product with sequence similarity to principal bacterially-produced toxins, such as cereus toxin (Choi *et al.*, 2009b). Instead, expression of the DNA inserts, when incorporated into *B. subtilis*, solely produce the desired product (*i.e.*, the 4-α-D-glucan branching enzyme). Therefore, *B. stearothermophilus* TRBE14 and *A. aeolicus* VF5 may be considered as safe sources of 1,4-α-D-glucan branching enzyme.

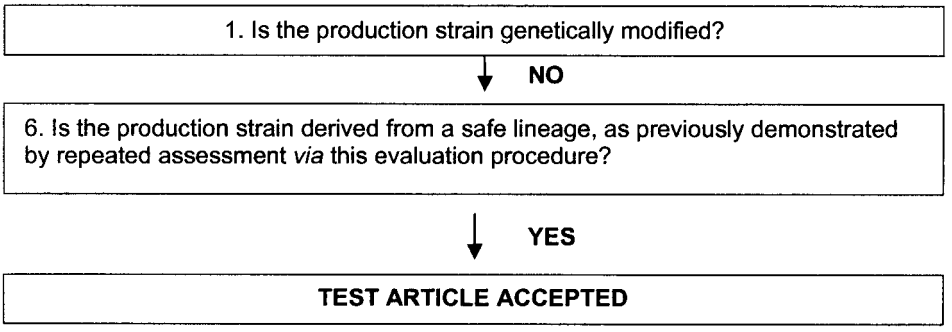
The information provided above also supports the safety of the DNA inserts for use in food processing. In addition, although 1,4-α-D-glucan branching enzyme does not have a documented history of use in food, a GRAS notice on 1,4-α-D-glucan branching enzyme produced from *B. subtilis* 168 expressing the 1,4-α-D-glucan branching enzyme gene from *Rhodothermus obamensis* (GRN 000274) has been filed by FDA with no questions (U.S. FDA, 2009), suggesting that the enzyme may be at present used in food production in the U.S. The self-affirmed GRAS determination of 1,4-α-D-glucan branching enzyme using the latter production strain supports the general safety of the microbial-derived branching enzyme for use in food production. Furthermore, branching enzymes are present naturally in plants, microorganisms, and animals, as well as in humans. For instance, 1,4-α-D-glucan branching enzyme occurs naturally in plants in which it is utilized for the formation of the amylopectin component of starch (Borovsky *et al.*, 1975, 1976). In humans (Thon *et al.*, 1993), animals (Huijing, 1975), and microorganisms (Preiss, 1984), the branching enzyme is utilized in the formation of glycogen.

The vector carrying the gene of interest is pUB110, a well-characterized vector that is documented 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). Although pUB110 harbors an antibiotic 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 food product. Thus, the pUB110 vector used to carry the genes encoding the branching enzyme is established as safe for constructing microorganisms to be used in the production of food products.

To ensure the safety of 1,4-α-D-glucan branching enzyme when produced using genetic modification techniques, the potential toxicity of BE-01 and BE-02 were formally assessed in subchronic oral toxicity studies and mutagenicity assays (see Section IV.E.2 and IV.E.3). Specifically, subchronic exposure to either BE-01 or BE-02 was not associated with any adverse effects of toxicological significance at doses of up to 870 mg/kg body weight/day in Sprague-Dawley rats. In addition, BE-01 and BE-02 were shown to be non-mutagenic in the bacterial reverse mutation assay (Ames test) using a range of bacterial strains both in the presence and absence of metabolic activation, and non-clastogenic in a chromosomal aberration assay conducted in mammalian cells [Chinese hamster ovary (CHO) cells]. Therefore, the DNA insert encoding the branching enzyme, when isolated from *B. stearothermophilus* or *A. aeolicus*, is safe for use in the manufacture of microbial enzyme preparations used in food production as the DNA encodes a safe protein product. The results of these studies also demonstrate the safety of the final branching enzyme preparation for use in food production.

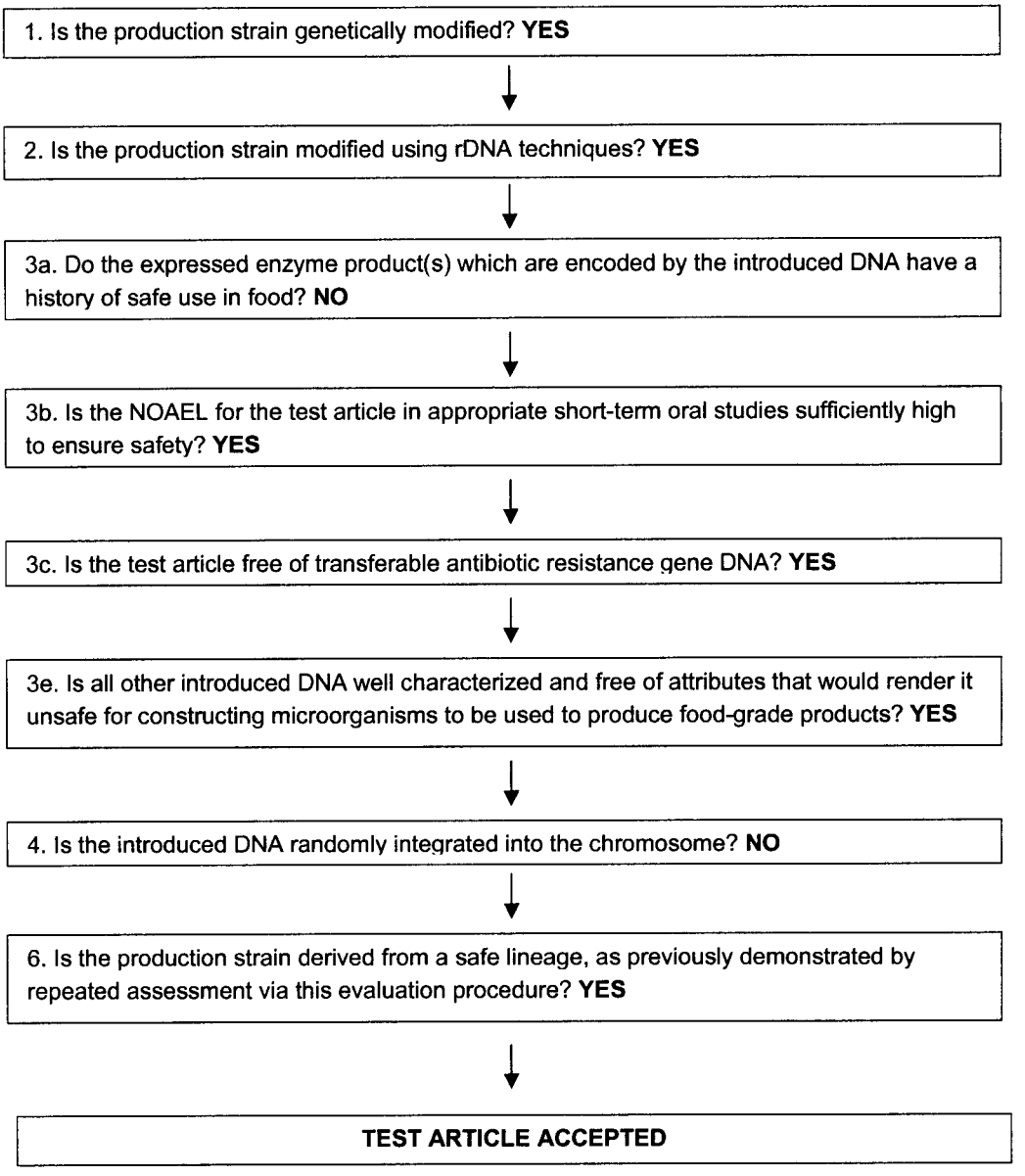
Figure IV.D-1 and IV.D-2 present the decision trees as they apply to the non-genetically-modified organism used in the production of BE (*B. stearothermophilus*) and the genetically-modified organisms used in the production of BE-01 and BE-02 (*B. subtilis*), respectively.

Figure IV.D-1 Decision Tree for Determining the Safety of *Bacillus stearothermophilus* for Use in Food



Modified from Pariza and Johnson (2001).

Figure IV.D-2 Decision Tree for Determining the Safety of Genetically-Modified *Bacillus subtilis* for Use in Food



Modified from Pariza and Johnson (2001).

IV.E Toxicological Studies

The results of product-specific toxicological studies support the safety of the final branching enzyme preparation when produced through any of the three production methods. Toxicological studies conducted using BE include an acute oral toxicity test and an *in vitro* mutagenicity test, as summarized in Section IV.E.1. Toxicological studies conducted using BE-01 and BE-02,

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which are summarized in Sections IV.E.2 and IV.E.3, respectively, include a 13-week subchronic toxicity test and *in vitro* mutagenicity and genotoxicity assays.

IV.E.1 1,4-α-D-Glucan Branching Enzyme Produced Using Non-Genetically-Modified *B. stearothermophilus* (BE)

IV.E.1.1 Acute Toxicity Studies

The acute toxicity of BE 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 BE by oral gavage following a 16-hour fast (Choi *et al.*, 2009a). This study was conducted in compliance with the Good Laboratory Practice (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 Ministry of Health and Welfare (MHW). The animals were fasted for a further 3 hours following administration of BE. For the 6 hours immediately following administration of BE, 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, as well as signs of toxicity, was assessed and documented. The body weight of the animals was recorded on Days 7 and 14 of the observational 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 BE 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. Furthermore, 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 BE, the oral LD_{50} of BE was considered to be greater than 2,000 mg/kg body weight. BE was further considered to have low acute toxicity following oral exposure.

IV.E.1.2 Genotoxicity Studies

The potential genotoxicity of BE was assessed in the L5178Y $TK^{+/-}$ mouse lymphoma forward mutation assay (Choi *et al.*, 2009a). This study was conducted in accordance with the MHW Guidelines for Toxicity Studies of Drugs and the OECD Guideline for the Testing of Chemicals Test No. 476, as well as in compliance with the MHW and OECD principles of GLP. 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 BE at concentrations of 156, 313, 625, 1,250, 2,500, or 5,000 µg/mL. BE 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 BE and the negative control either in the presence or absence of S9 metabolic activation. As expected, positive controls significantly induced the frequency of mutations compared to the negative control. Therefore, BE is non-genotoxic as assessed in the mouse lymphoma assay.

IV.E.2 1,4-α-D-Glucan Branching Enzyme Produced Using Genetically-Modified *Bacillus subtilis* Expressing the Branching Enzyme Gene from *B. stearothermophilus* (BE-01)

IV.E.2.1 Repeated Dose Studies

In order to examine the potential subchronic toxicity of BE-01, 5-week-old Sprague-Dawley [Crl:CD(SD)] rats (10/sex/group) were administered BE-01 at volumes of 1.7 (low-dose), 5 (mid-dose), or 15 (high-dose) mL/kg body weight/day by oral gavage for 13 weeks (Choi *et al.*, 2009b). This study was conducted in compliance with the MHW principles of GLP. Control animals were administered 15 mL of vehicle (water). The TOS value of the administered BE-01 test article was reported to be 58 mg/mL; therefore, the administered doses were equivalent to levels of 98.6, 290, and 870 mg/kg body weight/day, respectively, of BE-01. The animals were observed daily for general condition and mortality at least once before and after test article administration. Detailed clinical observations were recorded once before the first administration (on Day 1 of the study) and once a week thereafter. Body weights were measured on Day 1 of the study and once a week thereafter, as well as on the last day of administration. Food consumption was assessed the day before Day 1 of the study, on Day 2 of the study, and once per week thereafter, as well as on the last administration day. An ophthalmological examination was performed on 5 animals from each group in Week 13 of the study before test article administration. 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 animals from all groups survived the course of the study and appeared in good general condition. Body weights and food consumption throughout the course of the study did not differ significantly between any of the test groups compared to the control group. Ophthalmological examinations revealed normal results in all treated and control animals. Urinalysis results showed a significant increase in total excretion of sodium and chloride in male rats of the high-dose group compared to control males. In females of the high-dose group, a significant decrease in the total excretion of potassium was noted compared to control females. No other abnormalities were noted in any of the remaining urinalysis parameters examined. With the exception of a significant increase in the segmented neutrophil count in males of the high-dose group compared to controls, hematological parameters were similar among groups. Clinical

chemistry results indicated the following significant changes in males of the high-dose group compared to control males: increases in total protein, inorganic phosphorous, and β-globulin concentrations and decreases in the chloride concentration and the albumin/globulin ratio. In the females of the low-dose group, a significant increase in bile acid concentration compared to control females was detected and in females of the high-dose group, a significant decrease in blood glucose concentration was noted. However, the changes detected in females were not detected at the other doses. Results of all other serum chemistry parameters did not differ significantly between test and control groups.

Significant increases in the absolute and relative liver weights and a significant increase in relative kidney weights were observed in male animals of the high-dose group compared to controls. In addition, a significant decrease in the absolute kidney weights was observed in females of the low-dose group. Absolute or relative weights of all other examined organs were similar between test and control groups. Macroscopic examination of the organs revealed the occurrence of discoloration of the liver in rats of all test and control groups. Discoloration was observed in 3 males and 1 female in the control group, 3 male rats in the low-dose group, 4 males and 1 female in the mid-dose group, and 3 male rats in the high-dose group. Other than a diminishment in size of the left testis of 1 high-dose male rat and epididymis and right seminal vesicle of 1 male control rat, no gross abnormalities were observed in control or test animals. Due to the discoloration of the hepatic tissues, livers from all groups were subject to histopathological examination. Microscopic findings observed in the liver included focal hepatocellular necrosis, focal and perilobular hepatocellular vacuolation, microgranuloma, cholangiofibrosis, and infiltration by inflammatory and mononuclear cells in periductal and pericholangiolar areas, respectively; however, the incidences of these microscopic findings did not differ significantly between control and test animals. The frequency of all other histopathological observations was comparable between the control and test groups or only occurred in the control group and thus, these effects were not considered to be related to administration of BE-01.

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 as alterations were exclusively observed in only one gender and were not associated with any compound-related histopathological abnormalities. Specifically, urinary electrolyte alterations in the high-dose groups were not accompanied by compound-related increases in blood urea nitrogen or creatinine levels that would be indicative of impaired renal function or by histopathological variations in the kidney. Similarly, changes in kidney weights were not associated with renal abnormalities. Furthermore, alterations in serum chemistry parameters reported in the high-dose groups remained within the normal ranges of the laboratory's historical data. The most notable macroscopic and microscopic findings occurred in the liver. However, the frequency of the abnormalities did not differ significantly between test and control groups. No other compound-related abnormalities were identified in other tissues.

Based on their findings, Choi *et al.* (2009b) concluded that the no-observed-adverse-effect level (NOAEL) for BE-01 is 15 mL/kg body weight/day (approximately 870 mg/kg body weight/day), the highest dose tested, in experimental rats under the conditions of this study.

IV.E.2.2 Mutagenicity and Genotoxicity Studies

BE-01 was tested for potential mutagenicity in the bacterial reverse mutation assay (Ames test) using 4 *Salmonella typhimurium* strains (TA98, TA100, TA1535, and TA1537), as well as the *Escherichia coli* strain WP2*uvr*A in the presence or absence of metabolic activation using the plate-incorporation method (Choi *et al.*, 2009b). This study was conducted in compliance with the OECD principles of GLP, and in accordance with OECD Test Guideline No. 471. 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 metabolic activation: sodium azide, 9AA, 2-nitrofluorene, N-ethyl-N-nitrosourea, or 1-methyl-3-nitro-1-nitrosoguanidine. For assays conducted in the presence of metabolic activation, 2-AA or benzo[a]pyrene was employed as the positive control. Bacterial strains were treated with BE-01 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 BE-01 in the presence or absence of metabolic activation compared to the negative control. Treatment with BE-01 did not result in more than a 2-fold increase in the mean number of revertant colonies compared with the negative control at any concentration. In contrast, positive control agents substantially induced the number of revertant colonies compared to the negative control. Although 2-fold increases in the number of revertant colonies were not reached when compared to controls, higher levels of revertants were observed on TA98-, TA100-, TA1535-, and TA1537-treatment plates, together with a more dense background lawn (mainly with metabolic activation), an effect indicative of the presence of histidine or proteinous compounds in the test substance solution. Therefore, a second assay was performed according to the pre-incubation method in which the *S. typhimurium* strains were treated with BE-01 at concentrations of 312, 625, 1,250, 2,500, or 5,000 µg/plate. Dose-dependent cytotoxicity was not observed and BE-01 did not produce greater than 2-fold increases in the mean number of revertant colonies compared with the negative control in any strain of *S. typhimurium*. Therefore, BE-01 is non-mutagenic as assessed in the Ames test.

The genotoxic potential of BE-01 was further evaluated in a chromosomal aberration assay in cultured CHO cells with and without metabolic activation (Choi *et al.*, 2009b). This study was conducted in accordance with the OECD Test Guideline No. 473, and also to GLP standards. The culture medium served as the negative control. Mitomycin C (in the absence of S9) and cyclophosphamide (in the presence of S9) were employed as positive controls. In the first experiment, cells were treated with BE-01 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 BE-01. Cells co-incubated with BE-01 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 3 concentrations of BE-01 that induced cytotoxicity (between 1,250 and 5,000 µg/mL), along with the positive and negative controls, were further analyzed for chromosomal aberrations. The mitotic index was reduced in the absence of S9 metabolic activation upon continuous, but not pulse treatment, as well as in the presence of S9 (pulse treatment only) compared to the negative control at the 3 highest concentrations (≥1,250 µg/mL). Treatment with BE-01, however, did not significantly increase the number of aberrant cells (pulse- or continuously-treated) at these concentrations in the presence or absence of S9. The second experiment followed essentially the same methodology as the first. The final concentrations of BE-01 applied in the second set of experiments were based on the results of the first assay and were as follows: 500, 750, 1,000, 1,500, 2,000, 3,000, 4,000, or 5,000 µg/mL. The mitotic index was reduced at the 3 highest concentrations (≥3,000 µg/mL) by continuous treatment with BE-01, as well as by pulse treatment, but only upon prolonged incubation. However, treatment with BE-01 did not significantly increase the number of aberrant cells at these concentrations compared to the negative control. In both experiments, positive controls significantly induced the incidence of structural chromosomal aberrations compared to the negative control.

IV.E.3 1,4-α-D-Glucan Branching Enzyme Produced Using Genetically-Modified *Bacillus subtilis* Expressing the Branching Enzyme Gene from *Aquifex aeolicus* (BE-02)

IV.E.3.1 Repeated Dose Studies

A 13-week study was conducted to assess the safety of BE-02 in which 5-week-old Sprague-Dawley [Crl:CD(SD)] rats (10/sex/group) were administered BE-02 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.*, 2009b). This study was conducted in compliance with the MHW principles of GLP. Control animals were administered 15 mL of vehicle (water). The administered BE-02 test article was reported to have a TOS value of 60 mg/mL and therefore, the administered doses were equivalent to levels of 98.6, 290, and 870 mg/kg body weight/day, respectively, of BE-02. 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 BE-02. 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 BE-02.

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. As in the 13-week study with BE-01, 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 BE-02.

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 BE-02 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 other compound-related abnormalities were noted in other tissues. Therefore, the investigators concluded that under the conditions of this study, the NOAEL for BE-02 is 15 mL/kg body weight/day (approximately 900 mg/kg body weight/day) in experimental rats, the highest dose tested.

IV.E.3.2 Mutagenicity and Genotoxicity Studies

An Ames test was conducted to assess the potential mutagenicity of BE-02 at concentrations of 312, 625, 1,250, 2,500, or 5,000 µg/plate in *S. typhimurium* TA98, TA100, TA1535, and TA1537 and *E. coli* WP2*uvr*A (Choi *et al.*, 2009b). This study was conducted in compliance with the OECD principles of GLP, and in accordance with OECD Guideline for Test No. 471. The experiment followed the same procedure as that described for the assessment of the potential mutagenicity of BE-01 (see Section IV.E.2.2). No inhibition of bacterial growth was observed for any of the strains treated with BE-02 in the presence or absence of metabolic activation compared to the negative control. Treatment with BE-02 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. As expected, positive control agents substantially induced the number of revertant colonies compared to the negative control. Therefore, BE-02 is non-genotoxic as assessed in the Ames test.

The genotoxic potential of BE-02 was further evaluated in a chromosomal aberration assay following a similar methodology as that described for BE-01 (Choi *et al.*, 2009b). This study was conducted in accordance with OECD Test Guideline No. 473, and was conducted to GLP standards. BE-02 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, BE-02 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, BE-02 is non-genotoxic as assessed in the chromosomal aberration assay.

IV.F Safety Evaluations

Recently, JECFA evaluated the safety of a 1,4-α-D-glucan branching enzyme preparation derived from *B. subtilis* expressing the branching enzyme gene from *R. 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[4] for the 1,4-α-D-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-D-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.

IV.G Additional Safety Considerations

IV.G.1 Potential Allergenicity

A search conducted of the NCBI's protein database using the Protein Basic Local Alignment Search Tool (Protein BLAST) program failed to identify any protein sequence similarity of 1,4-α-D-glucan branching enzyme with known bacterially-produced toxins or known food allergens. Moreover, as described in Section IV.C, the manufacturing processes for cyclic dextrin, highly branched and enzymatically-synthesized glycogen ingredients incorporate steps to ensure inactivation and removal of the branching enzyme. Thus, it is not expected that the branching enzyme, when used as a food processing aid, would cause allergic reactions.

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[4] 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).

Moreover, there have been no reported cases of allergic reactions from the consumption of a cyclic dextrin, highly branched-based sports drink in Japan.

IV.H Summary and Basis for GRAS Conclusion

The GRAS determination for the use of 1,4-α-D-glucan branching enzyme as a processing aid in food production is based on scientific procedures. 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] may be produced from one of the following three production strains/methods: non-genetically-modified *Bacillus stearothermophilus* TRBE14 or genetically-modified *Bacillus subtilis* 168 expressing the 1,4-α-D-glucan branching enzyme structural gene *glgB* from either *B. stearothermophilus* TRBE14 or *Aquifex aeolicus* VF5. As such, the branching enzyme is referred to as BE, BE-01, or BE-02, respectively, in order to identify the production strain/method. The resultant protein product performs the same catalytic activity irrespective of the production strain. Thus, the branching enzyme subject of this GRAS exemption claim is the same irrespective of the production method. Genetic modification of *B. subtilis* was performed using standard techniques and the branching enzyme preparation is manufactured to cGMP, using food-grade materials and using a quality-controlled fermentation and purification process. The final 1,4-α-D-glucan branching enzyme preparation meets appropriate food-grade specifications and complies with the current specifications for enzyme preparations laid out 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 enzyme preparation has also been compositionally characterized and analyzed for other enzymatic activities. With regards to the latter, the BE preparation was identified as harboring weak α-amylase and α-glucosidase activities. These activities are assessed for every lot of the BE branching enzyme preparation and are under strict control.

1,4-α-D-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 and enzymatically-synthesized glycogen produced from food-grade dextrin. The typical use level of the branching enzyme is 500 U/g substrate in the production of cyclic dextrin, highly branched and 5,000 U/g substrate in the production of enzymatically-synthesized glycogen. The cyclic dextrin, highly branched and enzymatically-synthesized glycogen ingredients are intended for use in a variety of traditional foods and beverages. As the branching enzyme is used only as a processing aid, the branching enzyme will not added directly to food. In addition, the manufacturing processes for the final food ingredients incorporate steps to ensure inactivation and removal of the branching enzyme. Thus, carry-over of the branching enzyme into the final food ingredient, and thus into the final food product, is negligible.

The safety of 1,4-α-D-glucan branching enzyme was first evaluated 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 strains. For production strains derived *via* genetic modification the following elements also were considered: 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. Both *B. stearothermophilus* strain TRBE14 and *B. subtilis* are 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. Considering that *B. stearothermophilus* is non-toxigenic and non-pathogenic, the transfer of the *glgB* gene from *B. stearothermophilus* TRBE14 is considered safe for transfer into *B. subtilis* for the purposes of producing 1,4-α-D-glucan branching enzyme. The transfer of the *glgB* gene from *A. aeolicus* strain VF5, a Gram-negative bacterium, also is considered safe in the manufacture of the branching enzyme as no evidence indicates that strain harbors pathogenic or toxigenic potential. The DNA encoding the branching enzyme (the *glgB* gene) from either *B. stearothermophilus* TRBE14 or *A. aeolicus* VF5 do not encode for a product with sequence similarity to principal bacterially-produced toxins, such as cereus toxin (Choi *et al.*, 2009b). Instead, expression of the DNA inserts, when incorporated into *B. subtilis*, solely produce the desired product (*i.e.*, the 4-α-D-glucan branching enzyme). This information in addition to the GRAS exemption claim for 1,4-α-D-glucan branching enzyme derived from a different production strain (GRN 000274) 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, 1,4-α-D-glucan branching enzyme is considered safe for use in food production.

The safety of 1,4-α-D-glucan branching enzyme also has been evaluated in standard toxicological tests, specifically in acute or subchronic oral toxicity studies and in *in vitro* mutagenicity/genotoxicity assays. BE 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.*, 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 each of BE-01 and BE-02. Furthermore, BE 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.*, 2009a). BE-01 and BE-02 also were 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.*, 2009b). Collectively, the results of these studies indicate that the use of 1,4-α-D-glucan branching enzyme as a processing aid in food production would not be expected to produce adverse effects in humans when produced *via* any one of the three production methods.

Finally, the 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-D-glucan branching enzyme for use in the production of cyclic dextrin, highly branched and enzymatically-synthesized glycogen, produced consistently with cGMP and meeting appropriate food grade specifications described herein, are safe and suitable.

Furthermore, the Expert Panel unanimously concluded that the intended uses of 1,4-α-D-glucan branching enzyme are GRAS based on scientific procedures. It is also 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-D-glucan branching enzyme derived from non-genetically-modified *B. stearothermophilus* TRBE14 (BE) or genetically-modified *B. subtilis* 168 (BE-01 and BE-02) 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 and enzymatically-synthesized glycogen; therefore, it is excluded from the definition of a food additive 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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Table of CFR Sections Referenced (Title 21—Food and Drugs)

Part	Section §	Section Title
73—Listing of color additives exempt from certification	73.275	Dried algae meal
170—Food additives	170.30	Eligibility for classification as generally recognized as safe (GRAS)
172—Food additives permitted for direct addition to food for human consumption	172.340	Fish protein isolate
173—Secondary direct food additives permitted in food for human consumption	173.340	Defoaming agents
177—Indirect food additives: Polymers	177.1600	Polyethylene resins, carboxyl modified
	177.1655	Polysulfone resins
	177.2260	Filters, resin-bonded
	177.2510	Polyvinylidene fluoride resins
	177.2910	Ultra-filtration membranes
178—Indirect food additives: Adjuvants, production aids, and sanitizers	178.3297	Colorants for polymers
182—Substances generally recognized as safe	182.1	Substances that are generally recognized as safe
	182.1320	Glycerin
	182.6285	Dipotassium phosphate

Table of CFR Sections Referenced (Title 21—Food and Drugs)		
Part	**Section §**	**Section Title**
184—Direct food substances affirmed as generally recognized as safe	184.1027	Mixed carbohydrase and protease enzyme product
	184.1143	Ammonium sulfate
	184.1150	Bacterially-derived protease enzyme preparation
	184.1193	Calcium chloride
	184.1293	Ethyl alcohol
	184.1446	Manganese chloride
	184.1553	Peptones
	184.1742	Sodium carbonate
	184.1763	Sodium hydroxide
	184.1835	Sorbitol
	184.1854	Sucrose
	184.1857	Corn sugar
	184.1983	Bakers yeast extract

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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.

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27 July 2010
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APPENDIX B

APPENDIX B

METHODS OF ANALYSIS

Assay of activity of BE

This method is based on the ability of enzyme to decrease absorbance of amylase-iodine complex.

(1) Enzyme solution

The solution should be appropriately diluted so that the level of decrease of absorbance is within a range in which the decrease is proportional to concentration of enzyme.

The appropriate concentration of enzyme is from 15 to 30 units/mL.

(2) Substrate solution

Add 2 mL of 0.05mol/L potassium phosphate buffer (pH7.5) to 1 mL of amylose stock solution.

Dilute to 10 mL with distilled water.

(3) Iodine solution

Add 2 ml of 1 mol/L HCl to 0.5 mL of the iodine stock solution.

Dilutte to 260 ml with distilled water.

(4) Stop solution

Dilute 2 mL of 1 mol/L HCl to 500 ml with distilled water.

(5) Procedure

Put 0.1 mL substrate solution in a test tube, and incubate at 50±0.5℃ for 5 min.

Add 0.1 mL enzyme solution, and mix

Incubate accurately 10 min, add 2 mL of stop solution,

Add 2 mL of iodile solution, and mix thoroughly.

Determine the absorbance at 660 nm as A_B.

One unit of enzyme is defined as an amount which decrease the absorbance 0.05 % for 1min.

$$\text{Activity (unit/mL or g)} = \frac{A_B - A_T}{A_B} \times 100 \times \frac{1}{0.05} \times \frac{1}{10} \times \frac{1}{W}$$

Where,

A_T	:	Absorbance of test solution
A_B	:	Absorbance of blank solution
0.05	:	0.05%
10	:	Reaction time (min)
W	:	The amount of enzyme in 1 mL of test solution (g or mL)

(6) Reagent/solution

1) Amylose stock solution

Add 100 mL dimethylsulfoxide to 1.2 g of Amylose (Sigma, TypeIII), mix well, and incubated at 70℃ for 20 min.

Centrifuge at 10000× g for 10 min to remove insoluble substance.

Stock at 25 ℃

2) 0.05mol/L Potassium phosphate buffer (pH7.5)

Solution 1 : Dissolve 3.4g of potassium dihydrogenphosphate in water, and adjust to 500 mL with water.

Solution 2 : Dissolve 13.06g of dipotassium hydrogenphosphate in water, and adjust to 1500 mL with water.

Mix solutions 1 and 2 to adjust pH to 7.5.

3) Iodine stock solution

Dissolve 26g of KI in water, and add 2.6 g of I_2

Adjust to 100 mL with water.

Salmonella

Weighing	Aseptically weigh 25 g of the sample.
Pre-enrichment culture	Add 225 mL of EEM broth, mix well and incubate at 35°C for 18 hours.
Enrichment culture	Inoculate 1 mL of the pre-enrichment culture into Hajna-tetrathionate broth and incubate at 43°C for 18 hours.
Isolation	Streak a platinum loopful of the culture onto an XLD agar and a brilliant green agar plates, respectively, and incubate at 35°C for 24 hours.
Confirmation	Perform confirmatory testing on typical (or less typical) suspect colonies using the media below; for those showing typical features, identify serogroups by the slide agglutination test using O-group antisera. Confirmation tests • LIM medium • TSI medium • Simmons sodium citrate medium • Malonate medium
Labeling	Negative/25 g, Positive/25 g

Escherichia coli

Weighing	Aseptically weigh 10 g of the sample.
Preparation of sample solution	Add 90 mL of sterile phosphate buffered saline and mix well.
Primary culture	Inoculate 10 and 1 mL of the prepared sample solution and 1 mL of the 10-times diluted sample solution into 2 tubes each of BGLB broth fermentation tubes; incubate at 35°C for 2 days.
Secondary culture	Inoculate EC broth fermentation tube with a platinum loopful of the culture from BGLB broth fermentation tube that produced gas; incubate in a water bath set to 44.5°C for 24 hours.
Isolation	Streak a platinum loopful of the culture from EC broth fermentation tube that produced gas onto an EMB agar plate and incubate at 35°C for 24 hours.
Confirmation	Perform IMViC tests on typical colonies of the EMB agar medium using the media below. At the same time, transfer them onto nutrient agar slants and confirm that they are gram-negative non-spore-forming rods under a microscope.

IMViC tests

- SIM medium
- Glucose phosphate peptone medium
- Simmons sodium citrate medium

Labeling	Negative/2.22 g,	positive/1 g positive/0.1 g positive/0.01 g

Coliforms

BGLB method

Weighing	Aseptically weigh 10 g of the sample.
Preparation of sample solution	Add 90 mL of sterile phosphate buffered saline and mix well.
Inoculation	Inoculate 10 and 1 mL of the prepared sample solution and 1 mL of the 10-times diluted sample solution into 2 tubes each of BGLB broth fermentation tubes.
Culture	Incubate at 35°C for 2 days.
Isolation	Streak a platinum loopful of the culture from a BGLB broth fermentation tube that produced gas onto an EMB agar plate and incubate at 35°C for 24 hours.
Confirmation	Transfer typical and atypical suspect colonies on the EMB agar medium to lactose broth fermentation tubes and nutrient agar slants; perform the following confirmatory testing. • Confirmation of acid and gas production in a lactose broth fermentation tube • Confirmation of gram-negative non-spore-forming rods under a microscope
Labeling	Negative/2.22 g, positive/1 g positive/0.1 g positive/0.01 g

Total Viable Counts

Weighing	Aseptically weigh 10 g of the sample.
Preparation of sample solution	Add 90 mL of sterile phosphate buffered saline and mix well.
Dispensation	Dispense 0.5 mL of the prepared sample solution into each of 4 dishes. Also dispense 1 mL of its serial dilution into each of 2 dishes per dilution.
Pour plate	Pour approximately 15 mL of standard agar medium into plates, swirl to mix well, and allow it to solidify.
Culture	Incubate at 35°C for 2 days.
Count	Count the number of colonies grown after incubation.
Labeling	Multiply the colony count by the dilution factor to determine the total viable counts in CFU per 1 g of the sample.

June 2, 2010

Test for the production strains in the BE preparation

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Institute of Health Sciencces
Hiroki Takata

When microorganisms are detected in the sample, BE-01 and BE-02 are tested for the existence of production strains as follows. When the production strain is present in the test sample by, the lot will not be used.

1. Test for kanamycin-resistant bacteria.

One-hundred μL of enzyme preparation are plated on kanamycin-containing plate. The medium contains 1 % peptone, 0.5 % yeast extract, 0.5 % NaCl, and 10 μg/mL kanamycin. The plate is incubated at 37 °C for 48 h.
When any colonies appear, all of the bacteria are isolated and subjected to analyses for productions of BE (Test 2) and kanamycin-resistant genes (Test3).

2. Test for BE production

The kanamycin-resistant bacteria are separately cultivated in 10 mL-BE producing liquid medium. The medium contains 2.4% peptone, 2.88 % yeast extract, 0.13% potassium dihydrogen phosphate, 0.63 % dipotassium hydrogen phosphate, 0.0001 % $MnCl_2$, and 10 % sorbitol. After shaking at 37 °C for 48 h, 100 μL of the culture medium are mixed with 3.9 mL of lysis buffer, which include 0.03 mg/mL lysozyme, 0.05 % Triton X-100 in 10 mM potassium phosphate buffer (pH7.5). After incubation of the mixture at 37 °C for 2 h, BE activity is determined. If any activity would be detected, the production strain should be considered to be positive.

3. Test for kanamycin-resistant gene

The cells derived from the Test 2 are subjected to PCR analysis by using primers designed to detect kanamycin-resistant gene of pUB110. The cells of *Bacillus subtilis* NCIMB12378 (pUAQ2) is used as a positive control. If any DNA fragment would be detected, the production strain should be considered to be positive.

APPENDIX C

APPENDIX C

CERTIFICATES OF ANALYSIS AND COMPOSITIONAL DATA

EZAKI GLICO CO., LTD.

Certificate of Analysis

Product Name : BE (Branching Enzyme)

Lot No. : G015-02D

ITEMS	SPECIFICATIONS	RESULTS
Appearance	Dark-brownish liquid	Dark-brownish liquid
Activity (U/g)	≥10,000	32,600
Lead (ppm)	< 5	<0.05
Arsenic (as As_2O_3) (ppm)	≤4	<0.1
Total viable plate count (CFU/g)	≤10,000 CFU/g	1,800
Coliforms	Negative	Negative
Escherichia coli	Negative	Negative
Salmonella	Negative	Negative

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EZAKI GLICO CO., LTD.

Certificate of Analysis

Product Name : BE (Branching Enzyme)
Lot No. : G015-10D

ITEMS	SPECIFICATIONS	RESULTS
Appearance	Dark-brownish liquid	Dark-brownish liquid
Activity (U/g)	≥10,000	42,132
Lead (ppm)	< 5	<0.05
Arsenic (as As_2O_3) (ppm)	≤4	<0.1
Total viable plate count (CFU/g)	≤10,000 CFU/g	3,300
Coliforms	Negative	Negative
Escherichia coli	Negative	Negative
Salmonella	Negative	Negative

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EZAKI GLICO CO., LTD.

Certificate of Analysis

Product Name : BE (Branching Enzyme)

Lot No. : 8914037D

ITEMS	SPECIFICATIONS	RESULTS
Appearance	Dark-brownish liquid	Dark-brownish liquid
Activity (U/g)	≥10,000	30,656
Lead (ppm)	< 5	<0.05
Arsenic (as As_2O_3) (ppm)	≤4	<0.1
Total viable plate count (CFU/g)	≤10,000 CFU/g	4,900
Coliforms	Negative	Negative
Escherichia coli	Negative	Negative
Salmonella	Negative	Negative

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EZAKI GLICO CO., LTD.

Certificate of Analysis

Product Name : BE-01

Lot No. : 070910

ITEMS	SPECIFICATIONS	RESULTS
Appearance	Dark-brownish liquid	Dark-brownish liquid
Activity (U/g)	≥10,000	52,500
Lead (ppm)	< 5	<0.05
Arsenic (as As_2O_3) (ppm)	≤4	<0.1
Total viable plate count (CFU/g)	≤10,000 CFU/g	510
Coliforms	Negative	Negative
Escherichia coli	Negative	Negative
Salmonella	Negative	Negative
Production strain	Negative	Negative

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EZAKI GLICO CO., LTD.

Certificate of Analysis

Product Name : BE-01

Lot No. : 031016

ITEMS	SPECIFICATIONS	RESULTS
Appearance	Dark-brownish liquid	Dark-brownish liquid
Activity (U/g)	≥10,000	37,400
Lead (ppm)	< 5	<0.05
Arsenic (as As_2O_3) (ppm)	≤4	<0.1
Total viable plate count (CFU/g)	≤10,000 CFU/g	<300
Coliforms	Negative	Negative
Escherichia coli	Negative	Negative
Salmonella	Negative	Negative
Production strain	Negative	Negative

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EZAKI GLICO CO., LTD.

Certificate of Analysis

Product Name : BE-01

Lot No. : 030809

ITEMS	SPECIFICATIONS	RESULTS
Appearance	Dark-brownish liquid	Dark-brownish liquid
Activity (U/g)	≥10,000	36,200
Lead (ppm)	< 5	<0.05
Arsenic (as As_2O_3) (ppm)	≤4	<0.1
Total viable plate count (CFU/g)	≤10,000 CFU/g	940
Coliforms	Negative	Negative
Escherichia coli	Negative	Negative
Salmonella	Negative	Negative
Production strain	Negative	Negative

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EZAKI GLICO CO., LTD.

Certificate of Analysis

Product Name : BE-02 (AqBE)

Lot No. : 090915

ITEMS	SPECIFICATIONS	RESULTS
Appearance	Dark-brownish liquid	Dark-brownish liquid
Activity (U/g)	≥10,000	569,216
Lead (ppm)	< 5	<0.05
Arsenic (as As_2O_3) (ppm)	≤4	0.1
Total viable plate count (CFU/g)	≤10,000 CFU/g	<300
Coliforms	Negative	Negative
Escherichia coli	Negative	Negative
Salmonella	Negative	Negative
Production strain	Negative	Negative

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Glico

EZAKI GLICO CO., LTD.

Certificate of Analysis

Product Name : BE-02 (AqBE)

Lot No. : 060614

ITEMS	SPECIFICATIONS	RESULTS
Appearance	Dark-brownish liquid	Dark-brownish liquid
Activity (U/g)	≥10,000	535,294
Lead (ppm)	< 5	<0.05
Arsenic (as As_2O_3) (ppm)	≤4	<0.1
Total viable plate count (CFU/g)	≤10,000 CFU/g	<300
Coliforms	Negative	Negative
Escherichia coli	Negative	Negative
Salmonella	Negative	Negative
Production strain	Negative	Negative

Name Hiroki Takata

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EZAKI GLICO CO., LTD.

Certificate of Analysis

Product Name : BE-02 (AqBE)

Lot No. : 051221-2

ITEMS	SPECIFICATIONS	RESULTS
Appearance	Dark-brownish liquid	Dark-brownish liquid
Activity (U/g)	≥10,000	88,100
Lead (ppm)	< 5	<0.05
Arsenic (as As_2O_3) (ppm)	≤4	<0.1
Total viable plate count (CFU/g)	≤10,000 CFU/g	<300
Coliforms	Negative	Negative
Escherichia coli	Negative	Negative
Salmonella	Negative	Negative
Production strain	Negative	Negative

Name Hiroki Takata

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4/6/2010

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Institute of Health Sciences
Senior Researcher
Hiroki Takata, PhD

Report of Analysis

Product name BE

Items	Lot			Method of analysis
	G015-02D	G015-10D	8914037D	
Moisture	90.3%	90.2%	90.3%	Drying by heating
Protein and Nucleic acid	8.4%	7.3%	6.1%	Kjeldahl method
Fat	<0.1%	<0.1%	<0.1%	Acid hydrolysis method
Ash	<0.1%	0.3%	<0.1%	Direct ashing
Carbohydrate	1.3%	2.2%	3.6%	Calculation*1
Total Organic Solids	9.7%	9.5%	9.7%	Calculation*2

*1 Calculated as 100%-%Moisture-%Protein&Nculeic acid-%Fat-%Ash
*2 Calculated as 100%-%Moisture-%Ash

4/6/2010

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Institute of Health Sciences
Senior Researcher
Hiroki Takata, PhD

Report of Analysis

Product name BE-01

Items	Lot			Method of analysis
	070910	031016	030809	
Moisture	92.5%	92.0%	89.9%	Drying by heating
Protein and Nucleic acid	3.1%	2.6%	2.8%	Kjeldahl method
Fat	<0.1%	<0.1%	<0.1%	Acid hydrolysis method
Ash	0.9%	1.4%	1.9%	Direct ashing
Carbohydrate	3.5%	4.0%	5.4%	Calculation*1
Total Organic Solids	6.6%	6.6%	8.2%	Calculation*2

*1 Calculated as 100%-%Moisture-%Protein&Nculeic acid-%Fat-%Ash
*2 Calculated as 100%-%Moisture-%Ash

4/6/2010

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Institute of Health Sciences
Senior Researcher
Hiroki Takata, PhD

Report of Analysis

Product name BE-02 (AqBE)

Items	Lot			Method of analysis
	090915	060614	051221-2	
Moisture	93.2%	90.7%	92.6%	Drying by heating
Protein and Nucleic acid	2.2%	3.5%	2.8%	Kjeldahl method
Fat	<0.1%	<0.1%	<0.1%	Acid hydrolysis method
Ash	2.4%	2.4%	1.7%	Direct ashing
Carbohydrate	2.2%	3.4%	2.9%	Calculation*1
Total Organic Solids	4.4%	6.9%	5.7%	Calculation*2

*1 Calculated as 100%-%Moisture-%Protein&Nculeic acid-%Fat-%Ash
*2 Calculated as 100%-%Moisture-%Ash

SUBMISSION END

AM

Farias, Bianca

From: kuriki-takashi@glico.co.jp
Sent: Monday, November 08, 2010 11:45 PM
To: Ramos-Valle, Moraima
Cc: Ramos-Valle, Moraima; Farias, Bianca; takata-hiroki@glico.co.jp
Subject: RE: Fw: GRAS submission

Importance: High

Attachments: AUTHORIZATION for the contact person.pdf

AUTHORIZATION for the contact ...

Moraima J. Ramos Valle, M.S.
Consumer Safety Officer
Division of Biotechnology and GRAS Notice Review
Food and Drug Administration

Dear Moraima,

Thank you for your email.
I am sending my my statement authorizing Dr. Susan Choi as a contact person with FDA by attached pdf file.
If you need the hard copy, please let me know your mailing address. I am sending this via FEDEX as well.

All the best,
Tak

Takashi KURIKI, D. Eng.
Chief Scientific Officer
Director, Institute of Health Sciences
Ezaki Glico Co., Ltd.
4-6-5 Utajima, Nishiyodogawa-ku
Osaka 555-8502 Japan
Phone: +81-6-6477-8425
Fax: +81-6-6477-8362
Email: kuriki-takashi@glico.co.jp
http://www.glico.co.jp/en/index.htm

-----作成者: "Ramos-Valle, Moraima" <Moraima.Ramos-Valle@fda.hhs.gov> -----

>宛先: kuriki-takashi <kuriki-takashi@glico.co.jp>
>送信者: "Ramos-Valle, Moraima" <Moraima.Ramos-Valle@fda.hhs.gov>
>日付: 2010/11/09 01:22AM
>cc: "Ramos-Valle, Moraima" ,
>"Farias, Bianca" <Bianca.Farias@fda.hhs.gov>
>件名: RE: Fw: GRAS submission
>
>
>Dear Mr. Kuriki,
>
>We notice that in the submission Dr. Susan Choi is not listed as a
>contact person neither Cantox as an agent. In order for us to contact
>Dr. Choi with any issues regarding your GRAS submission for "1, 4- α
>-glucan branching enzyme" FDA will need a statement from you

000072

>authorizing us to talk to her or any other contact person not listed
>in the submission.
>
>Please let me know if you have any questions.
>
>Thanks,
>
>Moraima
>
>
>Moraima J. Ramos Valle, M.S.
>Consumer Safety Officer
>Division of Biotechnology and GRAS Notice Review
>Food and Drug Administration
>Phone: 301-436-1248
>Email: Moraima.Ramos-Valle@fda.hhs.gov
>
>
>
>
>
>From: Sharon Choi [mailto:schoi@cantox.com]
>Sent: Thursday, November 04, 2010 9:55 AM
>To: Ramos-Valle, Moraima
>Cc: kuriki-takashi; 高田 洋樹 ; 谷本秀典 ; takata-hiroki@glico.co.jp
>Subject: RE: Fw: GRAS submission
>
>Dear. Ms Ramos Valle,
>
>Thank you for bringing this to our attention. Would it suffice to
>send hardcopies of Page 1 (amended) only or is it required that we
>re-send hardcopies of the entire GRAS exemption claim, with Page 1
>amended. Please advise.
>
>Best regards,
>Sharon
>Sharon Choi, Ph.D.
>Scientific and Regulatory Consultant, Food and Nutrition
>Cantox Health Sciences International
>:Street :Street 2233 Argentia Road, Suite 308 :address
>Mississauga, ON L5N 2X7
>Tel: 905-542-2900
>Fax: 905-542-1011
>Email: schoi@cantox.com
>www.cantox.com / www.intertek.com
>
>
>****Draft of a mail to respond FDA***
>Mr. Moraima J. Ramos Valle, M.S.
>Consumer Safety Officer
>Division of Biotechnology and GRAS Notice Review Food and Drug
>Administration
>
>Dear Mr. Ramos Valle,
>
>Thank you for your mail dated on November 3, 2010 regarding our GRAS
>submissions.
>On behalf of Ezaki Glico, Cantox Health Sciences International will
>send the hard copies of the updated GRAS exemption claim.
>
>Sincerely,
>
Takashi Kuriki
>
>Takashi KURIKI, D. Eng.
>Chief Scientific Officer

>Director, Institute of Health Sciences
>Ezaki Glico Co., Ltd.
>4-6-5 Utajima, Nishiyodogawa-ku
>Osaka 555-8502 Japan
>Email: kuriki-takashi@glico.co.jp
>
>_____
>大阪市西淀川区歌島４－６－５
>**江崎グリコ株式会社**
>健康科学研究所　高田洋樹
>TEL 06-6477-8425
>FAX 06-6477-8362
>
>Hiroki Takata
>Ezaki Glico Co., Ltd.
>Institute of Health Sciences
>
>
>
>
>---------- **転送メッセージ** ----------
>From: <kuriki-takashi@glico.co.jp>
>日付 : 2010 年 11 月 4 日 9:29
>件名 : Fw: GRAS submission
>To: takata-hiroki@glico.co.jp
>
>
>
>
>**高田様**:
>**直ちに対応可能ですか？**
>栗木　拝
>----- 転送者 : 栗木隆 / **江崎** / **グリコ** 転送日 : 2010/11/04 09:29AM -----
>
>宛先 : "kuriki-takashi@glico.co.jp" <kuriki-takashi@glico.co.jp>
>送信者 : "Ramos-Valle, Moraima" <Moraima.Ramos-Valle@fda.hhs.gov>
>日付 : 2010/11/03 02:16AM
>cc: "Farias, Bianca" , "Martin, Robert L"
>
><Robert.Martin@fda.hhs.gov>
>件名 : GRAS submission
>
>Dear Mr. Kuriki,
>
>
>
>This message is to inform you that we are in receipt of the GRAS
>submissions for "cyclic dextrin" and "1, 4- α -glucan branching
>enzyme" both dated October 8, 2010.
>
>
>
>We noted that the GRAS exemption claim for the "1, 4- α -glucan
>branching enzyme" submission (page 1) do not clearly state who is
>making the GRAS determination, therefore making the claim incomplete.
>
>For that reason, we can not file this submission as it is, until we
>received an updated GRAS exemption claim stating that the notifier
>(Ezaki Glico Co., Ltd.) has determine that "1, 4- α -glucan branching
>enzyme" is GRAS, similarly to the GRAS exemption claim provided for
>the "cyclic dextrin" submission (page 3). You can send the hard
>copies of the updated GRAS exemption claim to the same address and to
>the attention of Dr. Robert L. Martin.

>
>
>
>Please feel free to contact me if you have any questions.
>
>
>Sincerely,
>
>
>
>Moraima J. Ramos Valle, M.S.
>
>Consumer Safety Officer
>
>Division of Biotechnology and GRAS Notice Review
>
>Food and Drug Administration
>
>Phone: 301-436-1248
>
>Email: Moraima.Ramos-Valle@fda.hhs.gov
(See attached file: AUTHORIZATION for the contact person.pdf)

Glico

AUTHORIZATION

Ezaki Glico Co., Ltd. authorizes the following person as a contact person with issues regarding our GRAS submissions for Cyclic Dextrin, Highly Branched, and 1,4-α-D-Glucan Branching Enzyme.

The contact person:

Dr. Sharon Choi
Scientific and Regulatory Consultant, Food and Nutrition
Cantox Health Sciences International
2233 Argentia Road, Suite 308
Mississauga, ON L5N 2X7, Canada

Authorized by:

(b) (6)

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

Date: November 9, 2010

AM

RECEIVED
NOV 12 2010
Division of
Biotechnology and
GRAS Notice Review

SENT *VIA* FEDEX

November 9, 2010

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: GRAS Notice for 1,4-α-D-Glucan Branching Enzyme

Dear Dr. Martin:

As requested, I am submitting in triplicate, as the notifier (Ezaki Glico Co., Ltd., 4-6-5, Utajima, Nishioyodogawa, Osaka, Japan], an amendment to the GRAS exemption claim statement (Section I, Pages 1 to 3) of the GRAS Notice recently submitted for 1,4-α-D-glucan branching enzyme.

Should you have any additional 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.

Sincerely,

(b) (6)

Takashi Kuriki
Chief Scientific Officer

Encl.

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NOV 12 2010
Division of
Biotechnology and
GRAS Notice Review

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-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) has been determined to be Generally Recognized as Safe (GRAS) by Ezaki Glico Co., Ltd. (Ezaki) 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 branching enzyme in food production as described below is exempt from the requirement of premarket approval.

Signed,

(b) (6)

November 10, 2010
Date

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

I.B Name and Address of Notifier

Takashi Kuriki
Chief Scientific Officer
Ezaki Glico Co., Ltd
4-6-5, Utajima, Nishioyodogawa
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-D-glucan branching enzyme.

I.D Conditions of Intended Use in Food

The branching enzyme is intended for use as a processing aid in food production. Specifically, the branching enzyme 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 and enzymatically-synthesized glycogen[2] produced from food-grade dextrin. The typical use level of the branching enzyme is 500 U/g substrate in the production of cyclic dextrin, highly branched and 5000 U/g substrate in the production of enzymatically-synthesized glycogen. The branching enzyme 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, 2010), 1,4-α-D-glucan branching enzyme has been determined to be GRAS based on scientific procedures for use in the production of cyclic dextrin, highly branched and enzymatically-synthesized glycogen.

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 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 Glico Co., Ltd. (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.
[2] A GRAS Exemption Claim for enzymatically-synthesized glycogen will be submitted to FDA in the near future.

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[3] (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-D-glucan branching enzyme, 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-D-Glucan branching enzyme 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 and enzymatically-synthesized glycogen; 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, Nishioyodogawa
Osaka, Japan

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

---

[3] 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 branching enzyme 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.

AM

Farias, Bianca

From: Sharon Choi [schoi@cantox.com]
Sent: Wednesday, November 10, 2010 9:29 AM
To: Ramos-Valle, Moraima
Cc: kuriki-takashi@glico.co.jp; Farias, Bianca; 高田 洋樹
Subject: RE: Fw: GRAS submission

Dear Moraima,

Many thanks for your prompt response. Ezaki Glico has sent the amended GRAS exemption claim statement (Section I, Pages 1 to 3 of the GRAS notice submitted) from their offices directly to FDA by courier.

Best regards,
Sharon

From: Ramos-Valle, Moraima [mailto:Moraima.Ramos-Valle@fda.hhs.gov]
Sent: November 9, 2010 9:11 AM
To: Sharon Choi
Cc: kuriki-takashi@glico.co.jp; Farias, Bianca
Subject: RE: Fw: GRAS submission

Dear Dr. Choi,

It will be helpful just to send the complete GRAS exemption claim section. That should be the first 3 or 4 pages that include the "GRAS exemption claim statement". Please let me know if you have any questions before sending this amendment to us.

Thanks,
Moraima

From: Sharon Choi [mailto:schoi@cantox.com]
Sent: Thursday, November 04, 2010 9:55 AM
To: Ramos-Valle, Moraima
Cc: kuriki-takashi; 高田 洋樹; 谷本秀典; takata-hiroki@glico.co.jp
Subject: RE: Fw: GRAS submission

Dear. Ms Ramos Valle,

Thank you for bringing this to our attention. Would it suffice to send hardcopies of Page 1 (amended) only or is it required that we re-send hardcopies of the entire GRAS exemption claim, with Page 1 amended. Please advise.

Best regards,
Sharon

Sharon Choi, Ph.D.
Scientific and Regulatory Consultant, Food and Nutrition
Cantox Health Sciences International

2233 Argentia Road, Suite 308
Mississauga, ON L5N 2X7
Tel: 905-542-2900
Fax: 905-542-1011
Email: schoi@cantox.com
www.cantox.com / www.intertek.com

****Draft of a mail to respond FDA***
Mr. Moraima J. Ramos Valle, M.S.
Consumer Safety Officer
Division of Biotechnology and GRAS Notice Review Food and Drug Administration

Dear Mr. Ramos Valle,

Thank you for your mail dated on November 3, 2010 regarding our GRAS submissions.
On behalf of Ezaki Glico, Cantox Health Sciences International will send the hard copies of the updated GRAS exemption claim.

Sincerely,

Takashi Kuriki

Takashi KURIKI, D. Eng.
Chief Scientific Officer
Director, Institute of Health Sciences
Ezaki Glico Co., Ltd.
4-6-5 Utajima, Nishiyodogawa-ku
Osaka 555-8502 Japan
Email: kuriki-takashi@glico.co.jp

大阪市西淀川区歌島４－６－５
江崎グリコ株式会社
健康科学研究所 高田洋樹
TEL 06-6477-8425
FAX 06-6477-8362

Hiroki Takata
Ezaki Glico Co., Ltd.
Institute of Health Sciences

---------- 転送メッセージ ----------
From: <kuriki-takashi@glico.co.jp>
日付: 2010年11月4日9:29
件名: Fw: GRAS submission
To: takata-hiroki@glico.co.jp

高田様：
直ちに対応可能ですか？
栗木 拝
-----転送者: 栗木隆/江崎/グリコ 転送日: 2010/11/04 09:29AM -----

宛先: "kuriki-takashi@glico.co.jp" <kuriki-takashi@glico.co.jp>
送信者: "Ramos-Valle, Moraima" <Moraima.Ramos-Valle@fda.hhs.gov>
日付: 2010/11/03 02:16AM
cc: "Farias, Bianca" <Bianca.Farias@fda.hhs.gov>, "Martin, Robert L" <Robert.Martin@fda.hhs.gov>
件名: GRAS submission

Dear Mr. Kuriki,

This message is to inform you that we are in receipt of the GRAS submissions for "cyclic dextrin" and "1, 4- α -glucan branching enzyme" both dated October 8, 2010.

We noted that the GRAS exemption claim for the "1, 4- α -glucan branching enzyme" submission (page 1) do not clearly state who is making the GRAS determination, therefore making the claim incomplete. For that reason, we can not file this submission as it is, until we received an updated GRAS exemption claim stating that the notifier (Ezaki Glico Co., Ltd.) has determine that "1, 4- α -glucan branching enzyme" is GRAS, similarly to the GRAS exemption claim provided for the "cyclic dextrin" submission (page 3). You can send the hard copies of the updated GRAS exemption claim to the same address and to the attention of Dr. Robert L. Martin.

Please feel free to contact me if you have any questions.

Sincerely,

Moraima J. Ramos Valle, M.S.

Consumer Safety Officer

Division of Biotechnology and GRAS Notice Review

Food and Drug Administration

Phone: 301-436-1248

Email: Moraima.Ramos-Valle@fda.hhs.gov

SUBMISSION END

2-14-10 Correspondence from Agent

AM

West-Barnette, Shayla

From: Sharon Choi Intertek [schoi@cantox.com]
Sent: Tuesday, December 14, 2010 11:51 AM
To: West-Barnette, Shayla
Subject: RE: Acknowledgement Letter for GRN 361 (subject: 1,4-Alpha-D-Glucan Branching Enzyme)

Dear Dr. West-Barnette,

A pleasure to hear from you. This is not a problem at all, since the notifier is keeping me up to speed, but thank you for the attachment. Please do continue to contact the notifier as well regarding this Notice.

Best regards,
Sharon

Sharon Choi, Ph.D.
Scientific and Regulatory Consultant, Food and Nutrition
Cantox Health Sciences International
An Intertek Company
2233 Argentia Road, Suite 308
Mississauga, ON L5N 2X7
Tel: 905-542-2900
Fax: 905-542-1011
Email: schoi@cantox.com
www.cantox.com / www.intertek.com

From: West-Barnette, Shayla [mailto:Shayla.WestBarnette@fda.hhs.gov]
Sent: December 14, 2010 10:44 AM
To: Sharon Choi Intertek
Subject: Acknowledgement Letter for GRN 361 (subject: 1,4-Alpha-D-Glucan Branching Enzyme)

Dear Dr. Choi,

I recently sent the acknowledgement letter for GRN 361 to Takashi Kuriki (the notifier). However, I have since become aware that Mr. Kuriki authorized you as a contact person for this notice. Thus, I have attached a copy of the acknowledgement letter to this message for your records. I will be sure to address and send future communications regarding this notice to you.

Please accept my apology for this error, and feel free to contact me if you need further assistance with this notice.

Regards,

Shayla West-Barnette, Ph.D.
Consumer Safety Officer
Center for Food Safety and Applied Nutrition
Food and Drug Administration
(301) 436-1262 (desk)
Shayla.WestBarnette@fda.hhs.gov

1-31-11 Correspondence from Notifier

AM

West-Barnette, Shayla

From: takata-hiroki@glico.co.jp
Sent: Monday, January 31, 2011 8:56 PM
To: West-Barnette, Shayla
Cc: Fus, Andrea *; stafazoli@cantox.com; kuriki-takashi@glico.co.jp; takata-hiroki@glico.co.jp
Subject: Withdrawal letter for FDA regarding GRN 361 and GRN 358

Attachments: FDA Withdrawal Letter for GRN358 and GRN361 Jan 31'2011.pdf

FDA Withdrawal Letter for GRN3...

Shayla West-Barnette, Ph.D.
Division of Biotechnology and GRAS Notice Review
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

CC: Dr. Andrea Fus, FDA
CC: Dr. Shahrzad Tafazoli, Cantox

Dear Dr. West-Barnette,

I am sending a letter for our request for withdrawal of the GRAS Exemption Notices for 1,4-alpha-D-Glucan Branching Enzyme Preparation (GRN 361) and Cyclic Dextrin, Highly Branched (GRN 358), on behalf of Takashi Kuriki, Chief Scientific Officer of Ezaki Glico Co., Ltd. Please find the attached file.

Thank you for your kind considerations on this matter.

Sincerely,

Hiroki Takata

Hiroki Takata, Ph.D.
Senior Researcher

4-6-5 Utajima, Nishiyodogawa, Osaka 555-8502, Japan.
Institute of Health Sciences
Ezaki Glico Co., Ltd.
E-mail: takata-hiroki@glico.co.jp
URL: http://www.glico.co.jp/en/index.htm

(See attached file: FDA Withdrawal Letter for GRN358 and GRN361 Jan 31'2011.pdf)

FDA Withdrawal Letter for GRN 358 and GRN 361 Jan 31' 2011.pdf
(attachment to 1-31-11 Correspondence from Notifier

Glico

EZAKI GLICO CO., LTD.

[illegible] CHOME UTAJIMA,
NISHIYODOGAWA-KU OSAKA JAPAN [illegible]
PHONE 06-6472-9306
FAX 06-6[illegible]-1604

SENT *VIA* EMAIL

January 31, 2011

Shayla West-Barnette, Ph.D.
Division of Biotechnology and GRAS Notice Review
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

Re: Withdrawal of Generally Recognized As Safe (GRAS) Exemption Notices for Alpha-D-Glucan Branching Enzyme Preparation and Cyclic Dextrin, Highly Branched

Dear Dr. West-Barnette:

This letter is to inform you that we would like to withdraw our GRAS Exemption Notices for 1,4-alpha-D-Glucan Branching Enzyme Preparation (GRN 361) and Cyclic Dextrin, Highly Branched (GRN 358), which were submitted to your office on October 8, 2010. This will allow us more time to adequately address the questions raised by the Agency regarding the aforementioned Notices.

Thank you for your kind attention to this matter. Please contact me should you have any questions regarding the withdrawal of these Notices.

Sincerely,

(b) (6)

Takashi Kuriki
Chief Scientific Officer