Glucoamylase from *Trichoderma reesei* expressed in *Trichoderma reesei*

Chemical and Technical Assessment (CTA)

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1. Summary

This Chemical and Technical Assessment summarizes data and information on the glucoamylase enzyme preparation submitted to JECFA by Genencor[1], a DuPont Industrial Biosciences Division, in a dossier dated January 07, 2013 (Genencor, 2013). This document also discusses published information relevant to glucoamylase, the Trichoderma reesei production organism, and the T. reesei organism that is the source of the glucoamylase gene.

Glucoamylase is an enzyme that catalyses the hydrolysis of terminal (1-4)-linked α-D-glucose residues successively from nonreducing ends with release of β-D-glucose in a polysaccharide substrate. It is used as a processing aid in the manufacture of corn syrups such as high fructose corn syrups (HFCS), in baking, brewing, and in the production of potable alcohol.

Glucoamylase is manufactured in a submerged straight-batch or pure culture fed-batch fermentation of a genetically modified strain of T. reesei containing the glucoamylase gene from T. reesei. T. reesei is considered to be a nonpathogenic and nontoxigenic fungus. It does not produce fungal toxins or antibiotics under conditions used for enzyme production. It has a long history of safe use in commercial enzyme production, including enzymes from genetically engineered strains of the organism.

The gene encoding glucoamylase was isolated from the wild-type T. reesei strain QM6a. T. reesei is classified as a Biosafety Level 1 (BSL1) microorganism by the American Tissue Culture Collection (ATCC). BSL1 microorganisms are not known to cause diseases in healthy humans. Authorities in Germany and Netherlands also consider T. reesei as a Risk Group 1 microorganism.

The production strain, 70H2-TrGA #32-9, was constructed via classical mutagenesis using two expression cassettes, to introduce the T. reesei gene encoding glucoamylase under the regulation of the native T. reesei cbhl (cellobiohydrolase 1) promoter and terminator, into the host strain, RL-P37. While one of the expression cassettes used the A. niduluns umdS gene as a selectable marker, the other used the endogenous T. reesei acetolactate synthase (ah) gene as a selectable marker. Both expression cassettes were integrated into the recipient chromosome.

Glucoamylase is secreted into the fermentation broth and is subsequently purified and concentrated. The final product is formulated and standardized to a desired activity and stability. The glucoamylase enzyme preparation complies with the General Specifications and Considerations for Enzyme Preparations Used in Food Processing (FAO/WHO, 2006).

Glucoamylase is not known to be allergenic when used in food processing. Nevertheless, comparison of the glucoamylase amino acid sequence to sequences of known allergens in the Structural Database of Allergenic Proteins (SDAP), using internationally accepted search criteria, did not indicate any meaningful identity. This demonstrates the inability of the enzyme to be a potential food allergen. Based on the results obtained, oral intake of the enzyme is not anticipated to pose any allergenicity concern.

2. Description

Amber liquid.

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3. Method of manufacture

3.1. T. reesei

T. reesei is a mesophilic filamentous fungus that is soil borne, and ubiquitous in nature. It was first isolated from cotton canvas in the Solomon Islands in 1944 (Kuhls et al., 1996). It has been classified as a BSL1 microorganism by the ATCC (i. e. not associated with diseases in healthy humans), and by culture collections in Germany (Deutsche Sammlung von Microorganismen und Zellkulturen, DSMZ) and Netherlands (Centraal Bureau Schimmelculturen, CBS). T. reesei is also considered a Class 1 Containment Agent under the National Institute of Health (NIH) Guidelines for Research Involving Recombinant DNA Molecules, and by the Ministerie van de Vlaamse Gemeenschap in Flanders, Belgium. It is considered as Good Industrial Large Scale Practice (GILSP) worldwide, and as a Risk Group 1 microorganism by authorities Germany and Netherlands. Also, it is not listed as a pathogen by EU (Directive Council Directive 90/679/EEC, as amended).

T. reesei has a long history of safe use in industrial-scale enzyme production including food, animal feed, pharmaceutical, textile and pulp and paper industries (Gallante et al. 1998; Olempska-Beer et al., 2006). It is non-pathogenic and has been shown not to produce fungal toxins or antibiotics under fermentation conditions used in enzyme production.

In 1988, JECFA evaluated cellulase from T. reesei and established an acceptable daily intake of “not specified” (FAO/WHO, 1988). T. reesei strains are in the Canadian Food and Drugs Act and Regulations (Table V of Division 16) as an authorized source for cellulase and pentosanase in several food applications. T. reesei (T. longibrachiatum var) strains are listed as a source of several carbohydrase enzymatic preparations (cellulase, beta glucanase, β-D-glucosidase, and hemicellulase) for use as processing aids in foods, in the 8th edition of “Food Chemicals Codex” (US Pharmacopeia, 2012). Cellulase from T. reesei, and glucoamylase from T. reesei, have been approved for use in brewing, baking, distilling and grain processing (journal number 2009-20-5406-00009) and brewing, baking, potable alcohol production, sweeteners and grain processing (journal number 2011-20-5406-00035/SMKE), respectively, in Denmark. In 1999, the U.S. Food and Drug Administration (US FDA) affirmed as Generally Recognized As Safe (GRAS) a cellulase enzyme preparation derived from a nonpathogenic and nontoxicogenic strain of T. longibrachiatum (now known as T. reesei) (21 CFR 184.1250). In recent years, US FDA has reviewed several GRAS notices for enzyme preparations derived from T. reesei, and has issued “no questions” letters in response to each of these notices. One notice, designated GRN 000372, discussed data and information supporting the safe use in food of the glucoamylase enzyme preparation from T. reesei expressing the glucoamylase gene from T. reesei, and is the subject of this Chemical and Technical Assessment.

3.2. T. reesei production strain

The glucoamylase-producing T. reesei strain was derived from the host strain, RL-P37. RL-P37 was developed from a wild-type parent T. reesei strain, QM6a via several classical mutagenesis steps. Several industrial cellulase productions are derived from QM6a. Though some Trichoderma species are known to be capable of producing various mycotoxins and antifungal metabolites, RL-P37 tested negative for mycotoxins and no antibiotic activity has been detected.

To construct the production strain, RL-P37 was modified to inactivate the genes encoding cellobiohydrolase 1 and 2, and endoglucanase 1 and 2, resulting in a strain with a compromised ability to use cellulose as a carbon source. The modified host strain was then transformed using two expression cassettes containing the glucoamylase gene from T. reesei. The first cassette contained the T. reesei cbh1 promoter and terminator, coding sequence for the endogenous genomic glal gene isolated from the parent host, QM6a, and the acetamidase gene from Aspergillus nidulans amdS gene, as a selectable marker for the transformation. The second cassette contained the T. reesei cbh

I promoter and terminator elements, glaI genomic DNA, and the T. reesei als gene encoding for acetolactate synthase as a selectable marker for the retransformation of one of the primary T. reesei transformants confirmed to express the first glucoamylase expression cassette. The recombinant process was evaluated to ensure successful incorporation of the genetic material. The final production strain, 70H2-TrGA #32-9, was tested to confirm copy number, and the absence of vector DNA used in the transformation of the host strain. The strain was also shown to be stable after culturing for more than 60 generations.

3.3. T. reesei donor strain

The source of the glucoamylase gene was T. reesei wild-type strain QM6a. This strain is present in several public culture collections, including ATCC (ATCC 13631). The glucoamylase gene, pNSP23, was isolated from QM6a by scientists at DuPont. Since QM6a is the parent strain of the host strain, RL-P37, the safety assessment of the host is applicable to the donor organism as well.

3.4. Fermentation, recovery, and formulation

Glucoamylase is produced in a submerged, aerobic, straight batch or fed-batch pure culture fermentation of the genetically modified T. reesei strain. The fermentation medium consists of food-grade raw materials to provide essential nutrients including glucose syrup, proteins such as soy meal and yeast extracts, various salts, and antifoam. The medium also contains compounds to control pH (aqueous ammonia or sulfuric acid). The fermentation process is carried out in sealed vessels designed to prevent release of the production organism and any contamination. The fermentation occurs at controlled temperatures. Upon completion of fermentation, the pH is adjusted, and the broth is cooled by diluting with water. The harvested liquid glucoamylase that is secreted into the fermentation broth is separated by filtration, centrifugation, or a combination of both processes, from the biomass that contains the production organism and spent fermentation media. The ensuing filtrate containing the enzyme is further concentrated to remove low molecular weight compounds. The resulting enzyme concentrate is subsequently diafiltered, and polished filtered, prior to measuring activity. This final enzyme concentrate is treated with sodium benzoate and potassium sorbate to achieve the desired glucoamylase stability.

4. Characterization

4.1. Glucoamylase

Glucoamylase catalyses the hydrolysis of terminal (1→4)-linked α-D-glucose residues successively from non-reducing ends of the chains with release of β-D-glucose from polysaccharide substrates. The Chemical Abstract Service Registry Number (CAS No.) of glucoamylase is 9032-08-0. Glucoamylase is classified by the Enzyme Commission of the International Union of Biochemistry and Molecular Biology (IUBMB, online edition) as follows:

Accepted name:	glucan 1, 4-α-glucosidase
Other name(s):	glucoamylase; amyloglucosidase; γ-amylase; lysosomal α-glucosidase; acid maltase; exo-1,4-α-glucosidase; glucose amylase; γ-1,4-glucan glucohydrolase; acid maltase; 1,4-α-D-glucan glucohydrolase
Reaction:	Hydrolysis of terminal (1→4)-linked α-D-glucose residues successively from nonreducing ends of chains with release of β-D-glucose
Systematic name:	4-α-D-glucan glucohydrolase
EC No.:	3.2.1.3

The activity of glucoamylase is measured relative to the glucoamylase standard using p-nitrophenyl-alpha-D-glucopyranoside (PNPG) as a substrate. The substrate is hydrolysed to glucose and p-nitrophenol. The absorbance of the yellow color formed by p-nitrophenol is measured at 400 nm. Color intensity is proportional to glucoamylase activity, which is expressed in GlucoAmylase Units (GAU). One GAU is defined as the amount of glucoamylase that liberates 1 g of reducing sugars equivalent to 5.6 mmole of glucose per hour, from soluble starch substrate at pH 4.3 and a temperature of 30°.

Glucoamylase has been evaluated for potential allergenicity using bioinformatics criteria recommended by the Codex Alimentarius Commission (FAO/WHO, 2003b). An amino acid sequence homology search between glucoamylase and known allergens listed in the SDAP database (http://fermi.utmb.edu/SDAP/index.html) was conducted. A primary search for 80-amino acid stretches with greater than 35% sequence identity yielded two matches with non-food allergens for the T. reesei GA; Pen ch 13 (fungi, Ascomycota Eurotiales; Penicillium chrysogenum, alkaline serine protease) and Lol p 5 (Poales; Lolium perenne, rye grass). No association with oral allergenicity has been reported specific to these two allergens. A secondary search for exact matches of short amino acid fragments that could serve as potential IgE binding sites resulted in no homology for sequence fragments of eight or more contiguous amino acids.

4.2. Glucoamylase enzyme preparation

The glucoamylase enzyme preparation is marketed as a liquid product under the trade name Diazyme. The composition of Diazyme is variable. A representative composition of a commercial formulation of glucoamylase enzyme preparation is provided below:

Total Organic Solids (TOS)	10-15.0%
Potassium sorbate	0.09-0.13%
Sodium benzoate	0.26-0.35%
Dextrose	10%
Water	79.65-84.52%

The TOS content is calculated according to the following equation:

$$\text{TOS}\ (\%) = 100 - (A + W + D)$$

where:

A = % ash, W = % water and D = % diluents and/or other formulation ingredients (NAS/NRC, 1981; FAO/WHO, 2006). TOS consists of the enzyme of interest and residues of organic materials, such as proteins, peptides, and carbohydrates, derived from the production organism and the manufacturing process.

The glucoamylase enzyme preparation conforms to the General Specifications and Considerations for Enzyme Preparations Used in Food Processing (FAO/WHO, 2006). It does not contain significant levels of secondary enzyme activities and is free from the production strain and transformable DNA.

5. Functional uses

The glucoamylase enzyme preparation is used as a processing aid in the manufacture of corn sweeteners such as HFCS, baking, brewing and potable alcohol manufacture. The action of this enzyme allows for the hydrolysis of terminal 1, 4-linked α-D-glucose residues successively from nonreducing ends of chains, with release of α-D-glucose from polysaccharide substrates.

The glucoamylase enzyme preparation is typically used in grain processing at levels ranging between 0.2 – 0.5 kg of Diazyme product per metric ton of starch on a dry weight basis. The enzyme preparation is used at 100-2000 mg/kg of flour weight in baking applications. In use as a processing aid for brewing and potable alcohol, a typical use level is 1.5 – 7.0 kg of glucoamylase enzyme preapration per metric ton of grist (e.g. malted barley) in mashing applications, and 0.3 - 0.5 kg of the enzyme preparation per metric ton of equivalent starch dry substance, in non-mashing applications.

6. Reactions and fate in food

The production of corn sweeteners, beer, and potable alcohol includes steps that would inactivate and/or remove glucoamylase. Thus, the carry-over of the active enzyme to food is expected to be negligible.

7. References

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