BRANCHING GLYCOSYLTRANSFERASE FROM *RHODOTHERMUS OBAMENSIS* EXPRESSED IN *BACILLUS SUBTILIS*
Chemical and Technical Assessment (CTA)

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1. Summary

This Chemical and Technical Assessment summarizes data and information on the branching glycosyltransferase enzyme preparation submitted to JECFA by Novozymes A/S[1] in a dossier dated 4 December 2008. This document also discusses published information relevant to branching glycosyltransferase, the production organism, *Bacillus subtilis*, and the source organism, *Rhodothermus obamensis*.

Branching glycosyltransferase is an enzyme that catalyses the transfer of a segment of a 1,4-α-D-glucan chain to a primary hydroxy group in a similar glucan chain to create 1,6-linkages thereby increasing the number of branched points. The branching glycosyltransferase described in the dossier is intended for use as a processing aid in the starch industry to obtain modified starch with improved functional properties such as higher solubility, lower viscosity, and reduced retrogradation. The modified starch is intended for use in the production of various foods, for example, sauces, dried instant food, low fat products, and soft drinks. Branching glycosyltansferase is expected to be inactivated and removed during starch processing steps following enzymatic modifications.

Branching glycosyltransferase is manufactured by pure culture fermentation of a genetically modified strain of *B. subtilis* containing a synthetic gene coding for branching glycosyltransferase from *R. obamensis*. *B. subtilis* is a Gram-positive bacterium that is widely distributed in nature and is considered to be nonpathogenic and nontoxigenic. *B. subtilis* has a long history of use in the production of enzymes used in food processing, including enzymes from genetically engineered strains.

The gene encoding branching glycosyltransferase was originally cloned from *R. obamensis*, a thermophilic bacterium that was isolated from a marine hydrothermal vent. Based on the amino acid sequence of branching glycosyltransferase translated from the *R. obamensis* gene, a synthetic gene was designed. The synthetic gene encodes branching glycosyltransferase with the same amino acid sequence as that of the native *R. obamensis* enzyme. The gene was subsequently placed under the control of appropriate DNA regulatory sequences and introduced into the *B. subtilis* host strain JA1343 by transformation. The chloramphenicol resistance gene (*cat*) was used in transformation as a selectable marker but it was subsequently deleted to make the production strain marker free.

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

Branching glycosyltransferase was assessed for potential allergenicity by comparing its amino acid sequence to the sequences of known allergens according to the bioinformatics criteria

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recommended in the Report of the Joint FAO/WHO Expert Consultation on Allergenicity of Foods Derived from Biotechnology (FAO/WHO, 2001). A 35% homology within a sliding window of 80 amino acids was identified to α-amylase from *Aspergillus oryzae*, which is recognized as an occupational allergen Asp o 21 and was also reported to cause allergy symptoms after ingestion in a few individuals. However, no homology between the branching glycosyltransferase and α-amylase from *A. oryzae* was found at the level of six contiguous amino acid sequences. In addition, branching glycosyltransferase is a bacterial protein, while nearly all known allergens are of eukaryotic origin. Thus, branching glycosyltransferase does not seem to have characteristics of a potential food allergen.

2. Description

Light brown liquid.

3. Method of manufacture

3.1. Bacillus subtilis

B. subtilis is a Gram-positive bacterium widely distributed in nature. It also occurs as a contaminant in human food and animal feed. *B. subtilis* is considered to be a nonpathogenic and nontoxigenic microorganism. It has a long history of safe use in research and in the production of enzymes and other compounds with food and medical applications. *B. subtilis* has been classified as a Risk Group 1 organism (i.e., not associated with disease in healthy adult humans) by the US National Institutes of Health (NIH, 2002). It has also been granted a Qualified Presumption of Safety (QPS) status by the European Food Safety Authority (EFSA, 2008).

The production strain for branching glycosyltransferase was derived from *B. subtilis* strain 168, a well characterized auxothrophic mutant with a fully sequenced genome. Numerous strains used in research and industrial applications have been developed from strain 168, including production strains for α-acetolactate decarboxylase and maltogenic amylase, both evaluated by JECFA (FAO/WHO, 1999).

3.2. Bacillus subtilis production strain

The host strain for branching glycosyltransferase, designated JA1343, was derived from strain 168 by replacement of four resident genes encoding sporulation factor F, neutral protease, alkaline protease, and amylase with inactive or deleted versions. Strain JA1343 is therefore sporulation negative, protease deficient and amylase negative.

The gene encoding branching glycosyltransferase was originally cloned from *R. obamensis*, a thermophilic bacterium isolated from a shallow hydrothermal vent in Tachibana Bay (Japan) (Sako et al., 1996). Based on the amino acid sequence of the *R. obamensis* branching glycosyltransferase, a synthetic gene was designed. The synthetic gene (named *BEK*) encodes branching glycosyltransferase identical to the *R. obamensis* enzyme.

The synthetic *BEK* gene was placed under DNA regulatory sequences derived from several *Bacillus* species and inserted into the chromosome of the *B. subtilis* host strain JA1343 at the *amyE* (amylase) locus. The chloramphenicol resistance gene (*cat*) was also initially inserted along with the *BEK* gene, but it was subsequently deleted with the help of a deletion plasmid to make the production strain marker free.

The recombinant production strain was tested for the stability of the introduced branching glycosyltransferase gene by Southern blot analysis of the genomic DNA isolated from the production strain before and after fermentation. The analysis confirmed the stability of the inserted branching glycosyltransferase gene.

3.3. Fermentation, recovery, and formulation

Branching glycosyltransferase is produced by submerged fed-batch pure culture fermentation of the genetically modified strain of *B. subtilis*. The fermentation medium consists of food-grade compounds providing adequate amounts of carbohydrates, proteins, minerals, and vitamins. The fermentation process is conducted under controlled conditions and monitored for microbial contamination. Branching glycosyltransferase is secreted to the fermentation broth and is subsequently purified and concentrated. The concentrated broth is subjected to pre- and germ filtration to remove the residual production strain and insoluble components of the fermentation broth. The enzyme concentrate is then stabilized with glycerol, sorbitol, and methionine, and formulated to a desired activity.

4. Characterization

4.1. Branching glycosyltransferase

Branching glycosyltransferase catalyses the transfer of a segment of a 1,4-α-D-glucan chain to a primary hydroxy group in a similar glucan chain to create 1,6-α-linkages. The Chemical Abstract Service Registry Number (CAS No.) of branching glycosyltransferase is 9001-97-2. Branching glycosyltransferase is classified by the Enzyme Commission of the International Union of Biochemistry and Molecular Biology (IUBMB, online edition) as follows:

Accepted name:	1,4-α-glucan branching enzyme
Other name(s):	branching enzyme; amylo-(1,4→1,6)-transglycosylase; Q-enzyme; α-glucan-branching glycosyltransferase, etc.
Reaction:	Transfers a segment of a (1,4)-α-D-glucan chain to a primary hydroxy group in a similar glucan chain
Systematic name:	(1,4)-α-D-glucan:(1,4)-α-D-glucan 6-α-D-[(1,4)-α-D-glucano]-transferase
EC:	2.4.1.18

The activity of branching glycosyltransferase is determined by measuring the rate of introduction of 1,6-α-linkages into the substrate amylose. The enzyme activity is expressed in branching enzyme units (BEUs). One BEU is defined as the quantity of enzyme that causes a decrease in absorbance at 660 nm of an amylose-iodine complex of 1% per minute under standard conditions (pH=7.2; 60^{o}).

Branching glycosyltransferase has been evaluated for potential allergenicity using bioinformatics criteria recommended by FAO/WHO (2001). An amino acid sequence homology search between branching glycosyltransferase and known allergens listed in the allergen database at http://fermi.utmb.edu/SDAP/index.html was conducted. No homology was found for sequence fragments of 6 contiguous amino acids. However, when using a sliding window of 80 amino acids, a 35% match was found to sequences of Asp o 21 allergen, which is the α-amylase from *Aspergillus oryzae* (TAKA amylase A). However, the sequence alignment of the two enzymes showed that there are large differences in the loop regions and the overall identity is only about 32%. Since the two enzymes belong to the same family of glycosylhydrolases (Family 13;

http://www.cazy.org/fam/GH13_3D.html), some homology is not surprising. Although α-amylase from *A. oryzae* is an occupational allergen (Skampstrup Hansen et al., 1999), allergy symptoms after ingestion of the enzyme were reported only for four individuals. Three of these individuals consumed bread baked with the enzyme (Baur and Czupon, 1995; Kanny and Moneret-Vautrin, 1995; Moreno-Ancillo et al., 2004)) and one had a positive response to the oral challenge with α-amylase (Losada et al., 1992). In other studies conducted with patients with documented occupational or other allergy, no cases of food allergy to α-amylase from *A. oryzae* and other commercial enzymes used in food were identified (Skampstrup Hansen et al., 1999; Bindslev-Jensen et al., 2006). Thus, food allergy to α-amylase from *A. oryzae* is extremely rare. Moreover, branching glycosyltransferase is a bacterial protein, while nearly all known allergens listed in allergen databases are eukaryotic proteins. Therefore, despite certain homology to α-amylase from *A. oryzae,* branching glycosyltransferase does not seem to have characteristics of a potential food allergen.

4.2. Branching glycosyltransferase enzyme preparation

The branching glycosyltransferase enzyme preparation is marketed as a liquid product and is standardized to 50 000 BEUs/g. The approximate composition of is:

Total Organic Solids (TOS)	4%
Water	44%
Sorbitol	25%
Glycerol	25%
Methionine	2%

The TOS content is calculated according to the following equation:

TOS (%) = 100 – (A + W + D)

where: A is % ash; W is % water; and D is % 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 branching glycosyltransferase 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 side activities and is free from the production strain.

5. Functional uses

The branching glycosyltransferase enzyme preparation is intended for use as a processing aid in the starch industry to produce modified starch with increased number of branch points and improved functional properties such as higher solubility, lower viscosity, and reduced retrogradation. The modified starch will be used in the production of various foods, for example, soups, sauces, dried instant food, low fat products, and soft drinks. The recommended use levels range from 0.4 to 40 kg enzyme preparation per ton of starch dry substance.

6. Reactions and fate in food

The branching glycosyltransferase enzyme preparation is intended for use in starch processing and will not be added directly to food. The reaction product formed during starch processing with the use of branching glycosyltransferase is modified starch containing 1,6-linkages typically present in a starch polymer amylopectin. The branching glycosyltransferase will be largely inactivated and removed during steps used in starch processing including inactivation of the enzyme (by heat or lowering pH), filtration, carbon treatment, ion exchange, evaporation, and drying. The carry-over of the active enzyme to food is expected to be negligible.

7. References

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