Hexose Oxidase from *Chondrus Crispus* expressed in *Hansenula Polymorpha*

CHEMICAL AND TECHNICAL ASSESSMENT (CTA)

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1 Summary

Hexose oxidase (HOX) is an enzyme that catalyses the oxidation of C6-sugars to their corresponding lactones. A hexose oxidase enzyme produced from a nonpathogenic and nontoxigenic genetically engineered strain of the yeast *Hansenula polymorpha* has been developed for use in several food applications. It is encoded by the hexose oxidase gene from the red alga *Chondrus crispus* that is not known to be pathogenic or toxigenic. *C. crispus* has a long history of use in food in Asia and is a source of carrageenan used in food as a stabilizer. As this alga is not feasible as a production organism for HOX, Danisco has inserted the HOX gene into the yeast *Hansenula polymorpha.* The information about this enzyme is provided in a dossier submitted to JECFA by Danisco, Inc. (Danisco, 2003).

Based on the hexose oxidase cDNA from *C. crispus,* a synthetic gene was constructed that is more suitable for expression in yeast. The synthetic gene encodes hexose oxidase with the same amino acid sequence as that of the native *C. crispus* enzyme. The synthetic gene was combined with regulatory sequences, promoter and terminator derived from *H. polymorpha*, and inserted into the well-known plasmid pBR322. The *URA3* gene from *Saccharomyces cerevisiae* (Baker's yeast) and the *HARS1* sequence from *H. polymorpha* were also inserted into the plasmid. The *URA3* gene serves as a selectable marker to identify cells containing the transformation vector. The native pBR322 plasmid contains genes encoding ampicillin and tetracycline resistance. These genes have been removed during the construction of the transformation vector.

The *H. polymorpha* production strain was developed from the wild-type strain ATCC 34438 by chemical mutagenesis. A strain requiring uracil for growth (a uracil auxotroph) was selected and used as a host strain. The strain was transformed with the hexose oxidase transformation vector and further improved using UV mutagenesis.

The hexose oxidase is produced by submerged fermentation of a pure culture of the *H. polymorpha* production strain. The enzyme is produced intracellularly and after cell disruption is released into the fermentation broth and subsequently separated from the yeast cells and subjected to ultrafiltration and diafiltration to obtain the concentrated hexose oxidase. Danisco is currently marketing the hexose oxidase product GRINDAMYL SUREBake 800.

The recombinant hexose oxidase is unstable at temperatures above 30^{o} and is expected to be inactivated during baking or pasteurization.

2 Description

The hexose oxidase preparation is an off-white to brownish microgranulate. It is prepared from the concentrated hexose oxidase by spray-drying onto a suitable food-grade carrier such as wheat starch.

3 Manufacturing

3.1 Hexose oxidase gene

The gene encoding hexose oxidase has been isolated from the alga *Chondrus crispus* in the form of cDNA (complementary DNA). *C. crispus* is the red alga that grows in the waters around North America and

Northern Europe. *C. crispus* is also known as "Irish moss" or "Carrageen seaweed." It is one of several seaweeds used as a raw material in the production of carrageenan used as a food stabilizer (http://www.seaweed.ie/). *C. crispus* and other related species have a long history of use in food in Asia. The sponsor performed a literature search on *C. crispus* and found no evidence of the alga being either pathogenic or toxigenic.

Based on the hexose oxidase cDNA, a synthetic gene was constructed to optimize the codon usage in the host organism *H. polymorpha*. Although certain changes were made in the DNA sequence, the optimized gene codes for hexose oxidase that has the same amino acid sequence as the wild type *C. crispus* enzyme.

3.2 Transformation vector

The transformation vector was constructed on the basis of the well-known *Escherichia coli* plasmid pBR322. The synthetic hexose oxidase gene was placed under the control of the FMD (formate dehydrogenase) promoter and the MOX (methanol oxidase) terminator, both derived from *H. polymorpha,* and inserted into the pBR322 plasmid. The *URA3* gene encoding orotidine-5-phosphate decarboxylase, derived from Bakers yeast *Saccharomyces cerevisiae,* and the *HARS1* sequence from *H. polymorpha* (*H. polymorpha* autonomous replicating sequence) were also inserted into the plasmid. In addition, the genes coding for ampicillin and tetracycline resistance, present in the original pBR322, were removed. The resulting plasmid was introduced into the *H. polymorpha* RB11 host strain using electroporation. The transformed strain was designated B13-HOX4.

3.3 The host Hansenula polymorpha

Background on Hansenula polymorpha

Hansenula polymorpha is a methylotrophic yeast capable of growing in a medium containing methanol as a sole carbon source. It can reproduce either by budding or by creation of ascospores that can germinate under appropriate nutritional conditions. *H. polymorpha* cells can either be haploid or diploid. Both forms can reproduce by budding, although diploid cells tend to sporulate easily. *H. polymorpha* is homothallic, and as such, is able to change the mating type and mate with itself by fusing two haploid cells with the opposite mating types. The resulting diploid cell can either make spores or reproduce by budding.

H. polymorpha occurs in soil rich in organic matter and in putrescent plant material. It was first described in 1951 (Wickerham, 1951) as *H. angusta.* Because of certain characteristics, such as the presence of strong promoters and the ability to secrete large amounts of proteins, *H. polymorpha* became a host for heterologous protein production. For example, a recombinant strain of *H. polymorpha* is used for the commercial production of hepatitis B vaccine.

There is no evidence in the literature that *H. polymorpha* is a pathogenic and toxigenic organism, although another species, *H. anomala* has been reported to be infectious in humans. However, one publication was found suggesting that *H. polymorpha* infected a chronically-ill child (McGinnis *et al*., 1980). This isolated case is not sufficient to support pathogenicity of *H. polymorpha*. However, it suggests that *H. polymorpha* acted as an "opportunistic pathogen." It is well-known that many microorganisms known as harmless, such as *S. cerevisiae* or *B. subtilis,* can cause opportunistic infections, i.e., infect immunocompromised individuals. The literature search did not reveal any reports associating *H. polymorpha* with allergenicity.

Construction of the host and production strains

The *H. polymorpha* wild type strain ATCC 34438 originally isolated from Brazilian soil was obtained from the American Type Culture Collection (ATCC). The strain was mutated using chemical mutagenesis. The mutant strain requiring uracil for growth (uracil auxotroph) was selected and used as a host strain for the hexose oxidase gene. The strain was designated RB11 and transformed with the transformation vector. A prototrophic transformant that does not require uracil (due to the presence of the *URA3* gene on the vector) was selected and designated as strain B13-HOX4. The strain was further improved using UV mutagenesis. The mutant strain was designated B13-HOX4-Mut45 and used as a hexose oxidase production strain.

Both B13-HOX4 and B13-HOX4-Mut45 strains were assessed for sporulation. Neither strain produced spores at measurable levels. Therefore, the strains are considered to be asporogenic.

The hexose oxidase production strain B13-HOX4-Mut45 contains multiple copies of the transformation vector integrated into the *H. polymorpha* genome.

Theoretically, if the transformation vector were integrated as tandem repeats, the insert can be unstable, as the copies of the vector may flip out of the genome by internal recombination and be transferred to other organisms. To test this possibility, the fermentation of the production strain was carried out for approximately 35 generations and the hexose oxidase activity was measured. The enzyme activity was steady throughout the experiment with the standard deviation at each point of less than 20%. Another similar experiment confirmed these results. The number of copies of the hexose oxidase gene has also been studied and found to be stable. These experiments indicate that the production strain B13-HOX4-Mut45 is stable with respect to the hexose oxidase production and the copy number of the hexose oxidase gene under the conditions tested.

The potential for survival and dispersal of the production strain outside the culture media used in hexose oxidase production was also tested. The growth of the B13-HOX4 strain on a rich medium containing yeast extract, peptone, and glycerol was studied at various temperatures from 5^{o} to 30^{o}. A very slow growth was observed above 15^{o} and no growth occurred below 15^{o}. Similar behaviour is expected for strain B13-HOX4-Mut45. The combination of slow growth under conditions that may be encountered in nature and asporogenicity minimizes the probability of survival and dispersal of the production strain under non-laboratory conditions.

4 Fermentation, recovery, and formulation

HOX is produced in a submerged fermentation process performed in fed-batch mode. After fermentation the process is terminated, product recovery is initiated. HOX is produced intracellularly in *H. polymorpha*. The cells are then disrupted with lauryl trimethyl ammonium bromide (LTAB) and the enzyme is released into the fermentation broth. The majority of the biomass is then removed by centrifugation. The almost cell-free liquid product stream from the centrifuge is filtered on a polishing filter to get a cell/debris-free product before ultrafiltration is initiated. The ultrafiltration increases the HOX concentration by a factor of approximately ten. The UF-concentrate is then diafiltered with water in a batch-wise mode. As a last step sterile filtration is performed. Finally the concentrate is spray dried onto wheat starch or other food grade carriers to form off-white to brownish microgranules.

5 Chemical characterization

5.1 Hexose oxidase

Hexose oxidase is described by the International Union of Biochemistry and Molecular Biology (IUBMB, Web Version) as follows:

Common name: hexose oxidase

Reaction: □-D-glucose + O_2 → D-glucono-1,5-lactone + H_2O_2

Systematic name: D-hexose:oxygen 1-oxidoreductase

The Chemical Abstract Service Registry number (the CAS No.) of hexose oxidase is 9028-75-5.

More than 48% of the amino acid sequence of the recombinant HOX has been determined by amino acid sequencing. All the obtained sequence data confirmed that the recombinant HOX is identical with the native HOX with regard to the amino acid sequence.

The enzyme activity is defined as HOX/g (hexose oxidase units/g). The HOX assay is based on measurement of hydrogen peroxide generated during the oxidation of glucose. The hydrogen peroxide is reduced with 2,2'-azino-bis(3-ethylbenzthiazolin-6-sulfonic acid) (ABTS) in presence of peroxidase to form a dye which is measured photometrically. One unit of enzyme activity is defined as the amount of enzyme which produces 1 μmol of H_2O_2 per min at 25 °C. The specific activity of HOX measured by this assay is approximately 100 HOX units/mg pure enzyme protein.

Some enzymes were reported to cause inhalation allergy in workers exposed to enzyme dust in manufacturing facilities. As other enzymes, hexose oxidase may also cause occupational allergy in sensitive

individuals. However, the possibility of an allergic reaction to the hexose oxidase residues in food seems remote. Danisco reports that in 1998 the Association of Manufacturers of Fermentation Enzyme Products (currently Association of Manufacturers and Formulators of Enzyme Products) Working Group on Consumer Allergy Risk from Enzyme Residues in Food performed an in-depth analysis of the allergenicity of enzyme products. The Group concluded that there were no scientific indications that small amounts of enzymes in food can sensitize or induce allergic reactions in consumers, and that the enzyme residues in foods do not represent any unacceptable risk to consumers (AMFEP).

5.2 Hexose oxidase preparation

Hexose oxidase is available as off-white to brownish microgranules. It is prepared from the concentrated hexose oxidase by spray-drying onto a suitable food-grade carrier such as wheat starch. The name of the commercial product is GRINDAMYL SUREBake 800. The product conforms to the General Specifications and Considerations for Enzyme Preparations Used in Food processing prepared at the 57th JECFA and published in FNP 52, Addendum 9 (JECFA, 2001). The product also conforms to the General and Additional Requirements set forth in the Food Chemicals Codex, 5th edition (FCC, 2003).

6 Functional use

HOX provides benefits in the production of a wide range of foods. It is able to catalyse the oxidation of a range of hexoses leading to the formation of lactones and hydrogen peroxide. The lactones will hydrolyse to the corresponding acids over time in aqueous systems.

When used in dough, HOX oxidizes mono- and disaccharides during formation of hydrogen peroxide leading to a strengthening of the gluten network.

Mono- and disaccharides are also oxidized in products like shredded cheese, potato chips, egg white powder and whey protein isolates. This limits the possibility of Maillard reactions, as the reducing sugar is no longer present in a form able to take part in this reaction. For shredded cheese, this gives added flexibility in the baking time for e.g. pizzas, as browning of the cheese is not as intensive. The use of an anti-sprouting agent, which prevents the formation of simple sugars, can be avoided in potatoes used to make potato chips. Egg white and whey protein powders can be dried without turning brown.

When used in cottage cheese and tofu, the acid formed by the oxidation of e.g. lactose acidifies the products and facilitates the precipitation of the protein/curd formation.

When used in products like ketchup, mayonnaise and salad dressings, HOX acts like an oxygen scavenger and eliminates dissolved oxygen in the products.

Hexose oxidase is used as a processing aid in the preparation of a wide range of foods at use levels from 150 up to 2,500 HOX Units per kg food.

7 Reactions and fate in food

The recombinant *Hansenula polymorpha* hexose oxidase is unstable at temperatures above 30^{o} and is expected to be inactivated during baking or pasteurization.

8 References

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