ISOAMYLASE FROM *PSEUDOMONAS AMYLODERAMOSA*
Chemical and Technical Assessment

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1. Summary

This Chemical and Technical Assessment summarizes data and information on the isoamylase enzyme preparation submitted to JECFA by Bioresco Ltd.[1] on behalf of Hayashibara Co. Ltd.[2] in a dossier dated December 19, 2006 (Hayashibara, 2006).

Isoamylase described in the Hayashibara's dossier catalyzes the hydrolysis of 1,6-α-D-glucosidic branch linkages in glycogen, amylopectin, and their β-limit dextrins. The enzyme is manufactured by pure culture fermentation of *Pseudomonas amyloderamosa* strain MU 1174. This production strain is a derivative of strain MI 414, which was developed by chemical mutagenesis from the wild-type strain SB-15. The SB-15 strain is deposited in the American Type Culture Collection under the accession number ATCC 21262. *P. amyloderamosa* is not known to be either pathogenic or toxigenic.

The *P. amyloderamosa* isoamylase is an extracellular enzyme that is secreted by the production strain to the fermentation broth. The fermentation broth is subsequently purified and concentrated to obtain isoamylase activity within a desired range. Isoamylase is then formulated with glucose and maltose and either glycerol n-caproate and glycerol octylate or sodium benzoate. Water is added to standardize isoamylase activity. The isoamylase enzyme preparation contains minor levels of cellulase, lipase, and protease activity.

Hayashibara manufactures two isoamylase products, "Isoamylase M" and "Isoamylase S." Both formulations contain approximately 37% of added sugars, glucose and maltose. Isoamylase M contains 0.05% of each, glycerol n-caproate and glycerol octylate, while Isoamylase S contains sodium benzoate instead of glycerol fatty acid esters.

The isoamylase enzyme preparation is intended for use in the production of food ingredients from starch, including glucose syrup, maltose, maltitol, trehalose, cyclodextrins, and resistant starch. The isoamylase enzyme preparation complies with the General Specifications and Considerations for Enzyme Preparations Used in Food Processing (JECFA, 2006).

2. Description

The isoamylase enzyme preparation is a yellowish brown liquid.

3. Method of Manufacture

3.1. Pseudomonas amyloderamosa *production strain*

P. amyloderamosa production strain MU 1174 is a derivative of strain MI 414, which was obtained from the wild-type strain SB-15 by chemical mutagenesis using N-methyl-N'-nitro-N-

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nitrosoguanidine. The SB-15 strain is deposited in the American Type Culture Collection under the accession number ATCC 21262. *P. amyloderamosa* SB-15 is a Gram-negative, non-sporulating, rod-shaped bacterium, which grows under aerobic conditions. The growth is optimal at 25-30^{o}C and pH 6.5-7.5.

P. amyloderamosa is a naturally-occurring soil microorganism, which secrets isoamylase into its extracellular environment (Harada et al., 1968; Sugimoto et al., 1974). It is not a human pathogen and is considered nontoxigenic. An extensive literature search performed by Hayashibara did not reveal any reports on adverse reactions to *P. amyloderamosa.* Hayashibara has used *P. amyloderamosa* in the production of isoamylase since 1979 without receiving reports of allergic or other adverse reactions to both *P. amyloderamosa* and the isoamylase enzyme preparation. To corroborate the safety of *P. amyloderamosa*, Hayashibara performed oral acute toxicity and pathogenicity tests in mice.

The oral acute toxicity test (LD_{50}) was performed with wet *P. amyloderamosa* cells and the filtrate of *P. amyloderamosa* culture (10 mice/sex/dose). No mortality occurred in either treatment. Hayashibara concluded that LD_{50} was greater than 66 g/kg for *P. amyloderamosa* cells and greater than 60 g/kg for the culture filtrate.

In the pathogenicity test, the suspension of *P. amyloderamosa* cells in physiological saline was intravenously administered to mice at three dose levels (5 mice/sex/dose). The highest dose was $4.35x10^{10}$ cells per mouse. After a 15-day observation period, the mice were killed and subjected to gross necropsy. Based on the results of this study, Hayashibara concluded that *P. amyloderamosa* does not induce acute toxic effects in mice and cannot reside and proliferate in the examined organs, i.e., the liver, kidneys, and brain.

3.2. Production Process

P. amyloderamosa strain MU 1174 is cultivated at about 28^{o}C in a standard medium containing food-grade ingredients, including dextrin, mineral salts, peptone or amino acids, and yeast extract. The fermentation conditions are monitored and the fermentation medium is periodically tested for microbial contamination. If contamination is detected, the fermentation batch is discarded. Isoamylase is an extracellular enzyme, which is secreted to the fermentation medium. When the desired isoamylase activity has been reached, the fermentation broth is filtered to remove the cellular debris. The enzyme solution is concentrated and purified by ultrafiltration and membrane filtration. Isoamylase is then formulated with food-grade glucose and maltose and either glycerol n-caproate and glycerol octylate or sodium benzoate. Water is added to achieve the intended isoamylase activity.

4. Characterization

4.1. Isoamylase

Isoamylase described in the subject dossier catalyzes the hydrolysis of 1,6-α-D-glucosidic branch linkages in glycogen, amylopectin, and their β-limit dextrins. Isoamylase does not catalyze the hydrolysis of pullulan, which is a linear polysaccharide consisting of maltotriose units linked by α-1,6-glycosidic linkages, and has a limited activity on α-limit dextrins. According to the Enzyme Commission of the International Union of Biochemistry and Molecular Biology, isoamylase is classified as follows (IUBMB, 2007):

Accepted name:	isoamylase
Reaction:	hydrolysis of (1→6)-α-D-glucosidic branch linkages in glycogen, amylopectin and their β-limit dextrins
Systematic name:	glycogen α-1,6-glucanohydrolase
Other name(s):	debranching enzyme
EC:	3.2.1.68

Other synonyms for isoamylase, not listed in the IUBMB monograph, are α-1,6-glucan hydrolase and glycogen-6-glucanohydrolase. The Chemical Abstract Service Registry Number (CAS No.) for isoamylase is 9067-73-6.

Isoamylase from *P. amyloderamosa* was initially identified and described by Harada et al., (1968). Its physico-chemical and molecular characteristics were subsequently elucidated and described in several publications (Yokobayashi et al., 1970; Yokobayashi et al., 1973; Kitagawa et al., 1975; Kainuma, et al., 1978; Katsuya et al., 1998). Isoamylase has a pH optimum at pH 3.0 – 4.0 and a temperature optimum at 52°C. The enzyme consists of 750 amino acid residues and has a molecular mass of about 80 kDa. The three-dimensional structure of isoamylase was elucidated by the X-ray structure analysis (Katsuya et al., 1998). The *P. amyloderamosa* gene *pmi* (also referred to as *ISO*) encoding isoamylase was cloned and sequenced (Amemura et al., 1988; Chen et al., 1990).

The isoamylase activity is determined by incubating the enzyme with soluble waxy corn starch as a substrate in the presence of iodine for 30 minutes under standard conditions (pH=3.5; 40°C) and measuring absorbance of the reaction mixture at 610 nm. The change in absorbance represents the degree of hydrolysis of the substrate. Isoamylase activity is calculated in isoamylase activity units (IAU) per gram of the enzyme preparation. One IAU is defined as the amount of isoamylase that increases absorbance of the reaction mixture by 0.008 in 30 minutes under standard conditions (pH=3.5; 40°C).

4.2. Isoamylase Enzyme Preparation

Hayashibara manufactures two isoamylase products, "Isoamylase M" and "Isoamylase S." Both products contain approximately 37% of sugars, glucose and maltose, added as stabilizers. Isoamylase M contains 0.05% of each, glycerol n-caproate and glycerol octylate, while Isoamylase S contains 0.1% of sodium benzoate instead of the glycerol fatty acid esters.

The isoamylase enzyme preparation contains minor levels of cellulase, lipase, and protease activity. The isoamylase activity in the final enzyme preparation is about 1,345,000 IAU/g. The typical composition of the isoamylase enzyme preparation is as follows:

Total Organic Solids (TOS):	2.5 – 6.6%
Water:	55.7 – 59.9%
Ash:	0.1 – 0.3%
Formulation ingredients:	37.3%

TOS is defined as:

TOS (%) = 100 – (A + W + D), where:

A = % ash, W = % water and D = % diluents and/or other formulation ingredients (JECFA, 2006).

The isoamylase enzyme preparation is tested to confirm compliance with the General Specifications and Considerations for Enzyme Preparations Used in Food Processing (JECFA, 2006) and to show the absence of viable cells of *P. amyloderamosa*. Hayashibara provided compliance data for three batches of the isoamylase preparation. The sponsor also tested three batches of the isoamylase preparation for several potential microbial contaminants and mycotoxins. One of the tested batches contained flat sour spores, which presumably entered the isoamylase preparation with the addition of the formulation ingredients, glucose and/or maltose. Flat sour spores are found in numerous food ingredients. They are formed by commonly-occurring food spoilage bacteria including *Bacillus subtilis, B. coagulans, B. licheniformis* and other species of the genus *Bacillus* (Richmond and Fields, 1966). None of the tested batches contained detectable levels of mycotoxins.

5. Functional Uses

The isoamylase enzyme preparation is used primarily in the production of food ingredients from starch. Isoamylase is typically used in combination with other starch-hydrolysing enzymes. Due to its debranching activity, isoamylase hydrolyses starch to linear dextrins, which may be subsequently degraded to α-1,4-linked gluco-oligosaccharides by α-amylase, maltose by β-amylase, or glucose by glucoamylase. Depending on the specific application, isoamylase is used at levels between 50-5000 IAU per gram of starch.

In the production of glucose syrup from starch, starch is liquefied using heat-resistant α-amylase. Subsequently, glucoamylase is typically added to convert the starch hydrolysate to glucose syrup. The addition of isoamylase from *P. amyloderamosa* results in a syrup with higher glucose content while reducing the amount of added glucoamylase (Norman, 1982).

Isoamylase can also be used in the production of maltose and maltitol. The enzyme is added to the liquefied starch after α-amylase has been inactivated by heat treatment. At the same time, β-amylase is added. The hydrolysis is carried out at an elevated temperature (50-55^{o}C) and a pH of about 5.0 and results in the formation of maltose syrup. The syrup is subsequently purified and concentrated and subjected to crystallization to obtain crystalline maltose. Maltose can then be converted to maltitol by the catalytic hydrogenation. Maltitol is used as a sugar substitute in the production of non-cariogenic hard candies, chewing gum, and other confectionary (Hirao et al., 1988).

Isoamylase is also used together with cyclodextrin glucanotransferase (CGTase), malto-oligosyl trehalose synthase, and malto-oligosyl trehalose trehalohydrolase in the production of a disaccharide trehalose from liquefied starch. The reaction product is trehalose syrup, which is subsequently purified and concentrated. Trehalose is used in food (for example, in bakery goods, beverages, confectionery, and breakfast cereals) as a texturizer, stabilizer, humectant, and sweetener.

Isoamylase can also be used in conjunction with CGTase to enhance the production of cyclodextrins from starch. For example, a 90% yield of β-cyclodextrin from amylopectin was obtained by applying a mixture of isoamylase, CGTase, and cyclodecanone at pH 6 and 25^{o}C. Cyclodecanone is a complexant that forms an insoluble inclusion complex with a cyclodextrin molecule (Rendelman, 1997). Cyclodextrins are used as encapsulating agents for food additives, flavours, and vitamins.

Poorly digestible or non-digestible starch (often referred to as resistant starch) may be obtained by processing gelatinized waxy maize starch, or low amylose potato starch, with isoamylase. After the reaction has been completed, the enzyme is inactivated either by heat or by lowering the pH to about 2.0. The pH is subsequently increased to 6-7 and the solution is kept at room temperature for 16 hrs. During that time, the debranched short-chain α-1,4-glucans retrograde and crystallize. The obtained crystalline cake of linear α-1,4-glucans is recovered by filtration and air-dried. The final product contains about 75% resistant starch that is not digested by pancreatic amylase at 37°C during 120 min (Shi et al., 2006).

6. Reactions and Fate in Food

As noted earlier, isoamylase is a debranching enzyme that catalyzes the hydrolysis of 1,6-α-D-glucosidic bonds in glycogen, amylopectin, and their β-limit dextrins. Isoamylase is either used or intended for use in the production of food ingredients derived from starch, such as glucose, maltose, trehalose, cyclodextrin, and resistant starch. Depending on the specific production process, isoamylase may be inactivated or its residues may be reduced or removed during the follow-up purification procedures that may include decolorization with active carbon, deionization with ion-exchangers, or crystallization.

7. References

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