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Isoamylase
GRAS Notification

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FOOD ADDITIVE SAFETY

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June 15, 2001

Letter

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Fine Ingredients and Products for the Food, Health, Cosmetic and Pharmaceutical Industries

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September 19, 2001

Office of Food Additive Safety
Department of GRAS and
Biotechnology Notification Review HFS 200
Food and Drug Administration
200 C St. SW
Washington, DC 20204

Dear Sir or Madam:

Originally Submitted June 20, 2001

In accordance with the proposed rule for Substances Generally Recognized as Safe, which was published in the *Federal Register* at Vol. 62, No. 74 on April 17, 1997, Hayashibara International of Westminster, Colorado would like to submit notice of a claim that the use of Hayashibara isoamylase as a food ingredient is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act, because such use is GRAS.

Isoamylase is a naturally occurring debranching enzyme that is capable of cleaving amylose side chains by hydrolyzing the α-1,6-glucosidic linkages found in amylopectin and glycogen. In nature the enzyme is produced as an extracellular secretion by certain microorganisms to metabolize branched α-glucans. These organisms utilize the resulting maltodextrins for growth and reproduction. Isoamylase is also produced in mammals, including humans, by the pancreas and salivary glands, where it participates in the digestion of complex carbohydrates. Recently, isoamylase has been identified in several significant food plants, such as maize, rice, pea, and potato as an important part of the system responsible for amylopectin synthesis. Therefore, the enzyme or products of the enzyme are consumed with many common starch foods.

A GRAS Report in support of the safe use of isoamylase in foods was prepared by Hayashibara International, and was reviewed on November 16, 2000 by a Panel of Experts qualified by training and experience to assess the safety of food ingredients.

The experts concurred with Hayashibara International's determination that isoamylase is safe for general use in foods. A copy of the Expert Opinion is attached to this notice.

On February 5, 2001 Hayashibara International personnel presented the salient points of the GRAS Report, and the conclusions of the Expert Panel to officials in the Center for Food Safety, and Applied Nutrition (CFSAN). Comments from that meeting have been incorporated into this notice of a claim for premarket exemption. This Notification is based on a GRAS determination under proposed §170.36.

Hayashibara International has prepared a Notification document in triplicate, which accompanies this letter. Within the Notification certain specific parameters pertaining to the manufacturing process have been shaded to preserve confidentiality. The general manufacturing steps and critical control points, however, remain unshaded for the agency's review. If agency personnel require the proprietary information, please inform Hayashibara International in writing, and a clear copy will be supplied for internal review.

The company would appreciate notice of the receipt of this document, and looks forward to any comments the agency would care to make on the Notification. If you have any questions regarding the content of the Notification, you may reach Dr. Alan Richards at (303) 650-4590 at the number listed above.

Sincerely,

Dr. Alan Richards
Vice President and General Manager

CC: Mr. Katsuaki Hayashibara, Hayashibara Company, Ltd.
Dr. Linda Kahl, FDA
Dr. Michael DiNovi, FDA

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Claim

Hayashibara International Inc.
GRAS Notification

Introduction

Isoamylase is a food-grade enzyme, which hydrolyzes the 1,6-α-D-glucosidic branch linkages in glycogen, amylopectin, and their α-limit dextrins. The enzyme is derived from a non-pathogenic, non-toxigenic hyper-isoamylase-producing proprietary strain of *Pseudomonas amyloderamosa*. *Pseudomonas amyloderamosa* is a naturally occurring organism, which shows no growth at 37°C.

The parent strain of *Pseudomonas amyloderamosa* associated with the current commercial production of isoamylase was deposited in the American Type Culture Collection, and was designated ATCC 21262. The strain under review in the Notification was derived by classic NTG-mutation, and therefore the enzyme is not the production of a genetically modified organism.

Isoamylase has been formally named glycogen 6-glucanohydrolase. Its CAS and Enzyme Commission numbers are 9067-73-6 and EC. 3.2.1.68, respectively. The enzyme is produced in nature by those bacteria, plants, and mammals, which metabolize complex carbohydrates, such as amylopectin or glycogen.

The Hayashibara Company, Ltd. (Hayashibara) of Okayama, Japan developed the bacterial strain, and the method for producing isoamylase more than two decades ago. Modern food processing research has shown that isoamylase may have an expanded role as a food ingredient.

This Notification document contains the information required in proposed 170.36 to allow the FDA to evaluate whether the submitted notice provides a sufficient basis for a generally recognized as safe (GRAS) determination. Hayashibara International Inc., of Westminster, Colorado, is submitting the document, which is a wholly owned subsidiary of Hayashibara. Both companies will be referred to as "Hayashibara" in this Notification, unless a specific distinction is necessary.

In compliance with 21 CFR § 170.30, Hayashibara determined that Hayashibara isoamylase could be considered GRAS when used in accordance with current Good Manufacturing Practices. Hayashibara wishes to voluntarily notify the Center for Food Safety and Applied Nutrition (CFSAN) of that determination, and according to proposed § 170.36, the company is submitting the following GRAS exemption claim.

Hayashibara International, Inc. has prepared a GRAS Report, which forms the basis for the information found in this Notification. The company also commissioned a panel of experts (Expert Panel), qualified by scientific training and experience, to assess the safety of isoamylase as a food ingredient. The Expert Panel critically evaluated the isoamylase GRAS Report as well as other data and information relevant to the use and safety of this ingredient. In a meeting held on November 16, 2000, the Expert Panel concurred with the company's determination that Hayashibara isoamylase can be considered generally recognized as safe for general use in food. Based on the data and information contained in the Report and the opinion of the Expert Panel (which is attached to this notification), Hayashibara explicitly accepts responsibility for the GRAS determination of Hayashibara isoamylase.

Section I. GRAS Exemption Claim

Hayashibara International, Inc. hereby notifies the U.S. Food and Drug Administration that the use of Hayashibara isoamylase as a food ingredient is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act, because Hayashibara has determined that such use is GRAS.

1. Notifier:

   Hayashibara International Inc.
   8670 Wolff Court, Suite 200
   Westminster, Colorado, USA 80030
   Telephone: (303) 650-4590
   Fax: (303) 650-9860

2. Common or Usual Name:

   Isoamylase

3. Applicable Conditions of Use:

   Applications for isoamylase include general use in foods as a multiple-use direct additive. The ingredient should be used under the conditions of current Good Manufacturing Practice.

   In order to classify the various effects ingredients may have in food, FDA has published a list of 32 physical or technical functional effects for which direct food ingredients may be added to food. These are codified at 21 CFR §170.3 (o) (1-32). Applications for isoamylase are covered under the following term as listed in 21 CFR §170.3 (o).

(9) "Enzymes": Enzymes used to improve food processing and the quality of the finished food.

4. Basis of the GRAS Determination

The basis of the GRAS determination for Hayashibara isoamylase was the use of scientific procedures.

5. Availability of Data and Information and Key to References

The data and information that are the basis of the GRAS determination for Hayashibara isoamylase will be available for FDA review and copying at the address of the notifier listed above. The notifier will also be pleased to provide the agency with a copy of the GRAS Report, or any references contained therein, upon written request. Throughout this Notification, citations to the published literature, which were included in the GRAS Report, are denoted as follows: [Author (*et al*), Year, Tab (number) Volume (number)]. In order to facilitate review of this document a complete list of references from the isoamylase GRAS Report is included in Appendix 2 as a key.

6. Signature of an official for Hayashibara International Inc.

Official for Havashibara International Inc. Date

June 19, 2001

Alan Richards, Ph.D.
Vice President and General Manager

Notification

ISOAMYLASE GRAS NOTIFICATION

Introduction

Isoamylase is a naturally occurring enzyme that has been found in many different species of pro-and eukaryotes [Manners, 1971 Vol 3 Tab 47 and Evans, *et al.*, 1971 Vol 2 Tab 15]. The enzyme is termed a "de-branching enzyme" because it is capable of cleaving amylose side chains by hydrolyzing the α-1,6-glucosidic linkages found in amylopectin and glycogen [Yokobayashi, *et al.*, 1973 Vol 5 Tab 80]. In nature the enzyme is produced as an extracellular secretion by certain microorganisms to metabolize branched α-glucans. These organisms utilize the resulting maltodextrins for growth and reproduction [Norrman, *et al.*, 1975 Vol 4 Tab 51]. Isoamylase is also produced in mammals, including humans, by the pancreas and salivary glands, where it participates in the digestion of complex carbohydrates [Skude, 1975 Vol 4 Tab 59 and Carlier, *et al.*, 1973 Vol 2 Tab 11]. Recently, isoamylase has been identified as an important part of the system responsible for amylopectin synthesis in higher plants [Kubo, *et al.*, 1999 Vol 3 Tab 36 and Fujita, *et al.*, 1999 Vol 2 Tab 18]. While it is not known if all higher plants produce isoamylase, a significant body of work has shown that it is an important enzyme in several significant food plants, such as maize, rice, pea, and potato [Fujita, *et al.*, 1999 Vol 2 Tab 18 and Zhu, *et al.,* 1998 Vol 5 Tab 84] Several research groups have hypothesized that isoamylase takes part in "nutrient packing" during starch biosynthesis, and, therefore, is consumed with many common starch foods [Sun, *et al.*, 1999 Vol 4 Tab 66].

Commercially, isoamylase is utilized as part of an enzyme system in the production of glucose, maltose, and other oligosaccharides from starch [Abe, *et al.*, 1999 Vol 2 Tab 1]. It is also used extensively to determine the structure of starch and starch products, particularly where the goal is to develop modifications in starch structure, which may lead to specific functionalities in food [Bello-Perez, *et al.*, 1996a Vol 2 Tab 7, Madhusudhan, *et al.*, 1996 Vol 3 Tab 45, and Manelius, *et al.*, 1997 Vol 3 Tab 46].

The commercial isoamylase enzyme preparation that is the subject of this Notification is derived from *Pseudomonas amyloderamosa*, a non-pathogenic organism, which shows no growth at 37°C. The organism is

naturally occurring, and the enzyme is ubiquitous in organisms that metabolize complex carbohydrates, such as starch and glycogen.

Hayashibara isoamylase has had a long history of safe commercial use as a food-grade enzyme in countries, such as Japan. More than 300,000 kilograms of the enzyme have been used in the food industry over the last two decades. It has been utilized in the production of a variety of food ingredients, including a high-purity maltose drip for intravenous feeding.

The value of this enzyme to the food industry has been widely recognized. Studies on the utilization, functionality, preservation and yield of isoamylase have been ongoing for more than three decades. Investigators worldwide continue to search for higher producing bacterial strains, more efficient ways to recover and preserve the enzyme, and new applications for its use [Lin *et al.*, 1994 Vol 3 Tab 42, Harada, 1994 Vol 2 Tab 24, Lin *et al.*, 1995 Vol 3 Tab 41, Hisamatsu, *et al.*, 1996 Vol 3 Tab 27 and Lai, *et al.*, 1996 Vol 3 Tab 37].

The following sections contain data and information pertinent to the safety evaluation of Hayashibara isoamylase. This GRAS Notice is formatted according to the agency's guidance document, "Preferred Elements of an Enzyme GRAS Petition."

Section I. Identity

1. Source

   Pseudomonas amyloderamosa MU 1174

   This strain is a hyper-isoamylase-productive strain derived from *Pseudomonas amyloderamosa* ATCC 21262 by classic NTG-mutation [Pienta, *et al.*, 1996 Vol 4 Tab 55 and Hayashibara, 2000, unpublished data]. *Pseudomonas amyloderamosa* is a naturally occurring soil microorganism, which secretes isoamylase into its extracellular environment [Harada, *et al.*, 1968 Vol 2 Tab 21, and Sugimoto, *et al.*, 1974 Vol 4 Tab 65].

2. Chemical Name and Enzyme Commission Classification

Glycogen 6-glucanohydrolase
EC. 3.2.1.68

3. Common and/or Trade Names

Isoamylase

4. Chemical Abstract Service (CAS) Registry Number

9067-73-6

5. Properties

a. Enzymatic Function

Isoamylase is a de-branching enzyme, which hydrolyzes the 1,6-α-D-glucosidic branch linkages in glycogen, amylopectin, and their α-limit dextrins [Yokobayashi, *et al.*, 1969 Vol 5, Tab 78]. It has no reported action on the major α-(1→4)-D-glycosidic linkages of starch, and it is virtually inactive against pullulan [Bello-Perez, *et al.*, 1996a Vol 2 Tab 7 and Rahman, *et al.*, 1998 Vol 4 Tab 56].

Amylopectin is a complex molecule, whose fine structure is still being elucidated by means of enzymatic degradation studies [Bello-Perez, *et al.*, 1996b Vol 2 Tab 7]. Hisamatsu *et al.* described amylopectin molecules as being composed of short-amylose chains comprised of approximately 6 – 100 glucosyl residues [Hisamatsu, *et al.*, 1996 Vol 3 Tab 27]. These chains may be variously branched via α-(1→6) glucosyl linkages [Rahman, *et al.*, 1998 Vol 4 Tab 56]. Amylopectin from various starch sources have different features; however, the basic structure is generally consistent. Usually, there is a main chain with secondary branches connected by α-(1→6) glucosyl

linkages. These secondary branches also have tertiary branches connected by the same α-(1→6)-glucosyl linkages. Molecular weights range from 10^7 to 5×10^8, and the percentage of amylopectin in some of the more common starches ranges from 30 to essentially 100% [Whistler, *et al.,* 1997 Vol 5 Tab 77]. By comparison pullulan is a polymer of maltotriose (three glucose units bound together α-(1→4) linkages) that are attached to other maltotriose units via α-(1→6) bonds [Rahman, *et al.*, 1998 Vol 4 Tab 56].

Sun *et al.* classified de-branching enzymes (DBEs) into two classes: direct debranching enzymes, which hydrolyze α-1,6-gluosidic linkages of α-polyglucans, and indirect debranching enzymes, which hydrolyze the α-1,6 branches by the combined actions of 4-α-glucanotransferase and amylo-1, 6-glucosidase [Sun, *et al.*, 1999 Vol 4 Tab 66]. Debranching enzymes, which directly hydrolyze, the α-1,6-glucosidic bonds of α-polyglucans were further classified into two groups: the R-enzyme or pullulanase type (also referred to as α-limit dextrinase or limit dextrinase; {EC 3.2.1.41} and the isoamylase type {EC 3.2.1.68}. In this classification system the biochemical distinction between isoamylase and pullulanase was primarily based on substrate specificity [Sun, *et al.*, 1999 Vol 4 Tab 66].

The debranching actions of crystalline isoamylase derived from *Pseudomonas* strain SB-15 were studied by Harada *et al.* The scientists determined that the branching linkages in 1 gram of waxy maize amylopectin were completely hydrolyzed by 0.01 mg of the isoamylase in 20 hours [Harada, *et al.*, 1972 Vol 2 Tab 22]. The extent of de-branching was determined by measuring the increase in reducing power, the coloration when iodine was added to the solution containing the hydrolyzed waxy maize, and the chain length of the resulting digests [Harada, *et al.*, 1972 Vol 2 Tab 22]. 1.3 mg of pullulanase did not completely hydrolyze the same substrate in 24 hours. However, the study showed that 13 mg of pullulanase produced a complete hydrolysis in 10 hours [Harada, *et al.*, 1972 Vol 2 Tab 22].

In the same study, 0.027 mg of isoamylase produced a complete hydrolysis of the branching linkages in oyster glycogen in 24 hours. In comparison 29 mg of pullulanase hydrolyzed approximately 30% of the oyster glycogen linkages in the same length of time [Harada, *et al.*, 1972 Vol 2 Tab 22]. Analyses of the debranched glucans produced by isoamylase after various incubation periods suggested that the enzyme hydrolyzes both the inner and outer branching linkages of amylopectin. In contrast, the authors stated that pullulanase hydrolyzes the outer linkages of amylopectin effectively, but has little effect on the inner linkages.

Kainuma *et al.* studied the action of *Pseudomonas* isoamylase on various branched oligo- and polysaccharides [Kainuma, *et al.*, 1978 Vol 3 Tab 32]. The investigators found that, as with *Klebsiella aerogenes* pullulanase, *Pseudomonas* isoamylase was unable to cleave D-glucosyl stubs from branched saccharides. Further, the optimum polysaccharide substrates for *Pseudomonas* isoamylase were higher in molecular weight than those easily hydrolyzed by pullulanase. The authors found that the smallest units hydrolyzed by pullulanase were tetrasaccharides, whereas the smallest substrate capable of being hydrolyzed by *Pseudomonas* isoamylase was a pentasaccharide. *Pseudomonas* isoamylase was found to cleave oligosaccharides at a slower rate than amylopectin or glycogen under the experimental conditions of this study [Kainuma, *et al.*, 1978 Vol 3 Tab 32]. Kainuma *et al.* concluded that *Pseudomonas* isoamylase required a minimum of a maltotriose molecule for hydrolysis. They cited as an example, that a maltosyl branch in the A chain of an oligosaccharide was always cleaved at a lower rate than a maltotriosyl branch [Kainuma, *et al.*, 1978 Vol 3 Tab 32].

Isoamylases in Amylopectin Synthesis

Recently, isoamylases were identified as essential to the synthesis of amylopectin in certain higher plants used as food

sources, such as potatoes, maize, barley, and rice [Ishizaki, 1978 Vol 3 Tab 30, Rahman, *et al.*, 1998 Vol 4 Tab 56, Sun *et al.,* Vol 4 Tab 6 and Fujita, *et al.*, 1999 Vol 2 Tab 18]. Previously, the metabolic function of debranching enzymes was thought to be the degradation of amylopectin in storage starch during germination. New studies have suggested that isoamylase and pullulanase may play an essential role in amylopectin synthesis, in conjunction with starch synthases, and starch-branching enzymes [Fujita, *et al.*, 1999 Vol 2 Tab 18].

Ishizaki *et al.* was the first to report a debranching enzyme in higher plants, which was capable of hydrolyzing glycogen [Ishizaki, 1978 Vol 3 Tab 30]. The enzyme was purified from potato tubers (*Solanum tuberosum* L.), and had no activity against pullulan. Therefore, according to the author it was not an R-enzyme (pullulanase). After 20 hours of incubation with rabbit liver glycogen, 42% of the branch linkages were hydrolyzed. The extent of hydrolysis was calculated from the amount of reducing sugars produced and by measuring the average chain length (in this case 15 glucose units). Under the same conditions, 65% of the branch linkages in amylopectin were hydrolyzed by the enzyme [Ishizaki, 1978 Vol 3 Tab 30].

In a study published by Beatty *et al.* specific isoamylase- and pullulanase-type starch-debranching enzymes (DBEs) were identified and purified from developing maize (*Zea mays* L.) endosperm [Beatty, *et al.*, 1999 Vol 2 Tab 6]. The enzymes represented the two types of DBEs that have been identified in plants: pullulanases and isoamylases [Rahman, *et al.*, 1998 Vol 4 Tab 56]. Plant pullulanases hydrolyze both pullulan, and α-limit dextrins at a much higher rate than amylopectin [Rahman, *et al.*, 1998 Vol 4 Tab 56]. However, they have virtually no activity against glycogen. Isoamylases, on the other hand, readily hydrolyze the α-(1$\rightarrow$6) branch linkages of both amylopectin and glycogen, but do not have any significant activity against pullulan.

In the Beatty *et al.* study the cDNA clone zpu1 [small letters

denote a gene] was isolated based on its homology with a rice (*Oryza sativa* L.) cDNA coding for a pullulanase-type DBE. A comparison of the protein product, ZPU1 [capital letters denote the protein product of a gene], with 18 other DBEs revealed patterns common to both isoamylase- and pullulanase-type enzymes. The pullulanase-type activity was identified as ZPU1 and the isoamylase-type activity as SU1. Mutations [in Italics] of the sugary 1 (*su1*) gene were known to the authors to cause deficiencies of the SU1 isoamylase and a pullulanase-type DBE. The investigators found that ZPU1 activity, protein level, and electrophoretic mobility were altered in *su1*-mutant kernels, indicating that it is the affected pullulanase-type DBE. Since the zpu1 transcript levels were equivalent in non-mutant and su1-mutant kernels, it was suggested that the coordinated regulation of ZPU1 and SU1 occurs post-transcriptionally [Beatty, *et al.*, 1999 Vol 2 Tab 6].

Previous to the findings of Beatty *et al.* the role of isoamylase in amylopectin biosynthesis was investigated in sugary 1 (*su1*) mutant maize endosperm. Rahman *et al.* published a report in which the authors hypothesized that isoamylase was in part responsible for the structure of storage starch in maize endosperm. Normal function of the sugary 1 gene has been shown to be required for starch biosynthesis. However, endosperm-containing mutants that are homozygous for the *su1* mutation were known to accumulate phytoglycogen at the expense of amylopectin [Rahman, *et al.*, 1998 Vol 4 Tab 56].

These data suggested that both amylopectin and phytoglycogen shared a common precursor, and that the product of the *su1* gene, participated in kernel starch biosynthesis. The product of the gene (SU1) was found to be similar in sequence to α-(1$\rightarrow$6) glucan hydrolases (starch-debranching enzymes). Specific antibodies were used to demonstrate that SU1 was a 79-kD protein that accumulated in endosperm during starch biosynthesis [Rahman, *et al.*, 1998 Vol 4 Tab 56]. A nearly complete SU 1 protein was expressed in *Escherichia coli* and purified. Two

different biochemical assays confirmed that SU1 hydrolyzed α-(l→6) linkages in branched polysaccharides. Determination of the specific activity toward various substrates enabled the investigators to classify the protein as an isoamylase. The authors indicated that previous studies had shown, however, that *su1*–mutant endosperm were also deficient in a second type of DBE, a pullulanase (or R enzyme). Immunoblot analyses revealed that both SU1 and a protein detected by antibodies specific for the rice (*Oryza sativa*) R enzyme were missing from *su1*-mutant kernels. The authors concluded that their data supported the hypothesis that DBEs are directly involved in starch biosynthesis [Rahman, *et al.*, 1998 Vol 4 Tab 56].

According to Rahman *et al.* physical and chemical analyses of granular starch have led to a widely accepted model for amylopectin structure called the "cluster model," in which amorphous and crystalline regions alternate with a defined periodicity [Rahman, *et al.*, 1998 Vol 4 Tab 56]. Within amylopectin the crystalline component is composed of parallel arrays of linear chains packed tightly in double helices. Branch linkages, which account for approximately 5% of the glucosyl linkages in amylopectin, are located at the root of each cluster in the amorphous region. This periodic clustering of branches allows for the alignment of the linear chains and their dense packing into crystalline regions. Chain packing provides an efficient mechanism for nutrient storage. Rahman *et al.* speculated that the enzymatic processes required to create such an ordered spatial arrangement of branch linkages must be highly regulated and coordinated [Rahman, *et al.*, 1998 Vol 4 Tab 56]. The suggestion that DBEs participate in the organization of regularly spaced clusters within amylopectin, was thought to be strengthened by the finding that phytoglycogen accumulates in *su1*-mutant kernels.

Zeeman *et al.* also observed that a mutant of the plant, *Arabidopsis*, which lacked a chloroplastic isoamylase, accumulated both starch and phytoglycogen [Zeeman, *et al.*, 1998

Vol 5 Tab 81]. The goal of the Zeeman *et al.* study was to evaluate the role of starch debranching enzymes in the determination of the structure of amylopectin. Using iodine staining, mutant populations of *Arabidopsis* were screened for plants with alterations in the structure of leaf starch. The leaves of two mutant lines stained reddish brown, whereas wild-type leaves stained brownish black, indicating that a more highly branched polyglucan than amylopectin was present. The mutants were allelic, and one mutant line lacked the transcript for a gene with a sequence similar to higher plant debranching enzymes. Both mutants lacked a chloroplastic starch-hydrolyzing enzyme. The authors identified the protein as a debranching enzyme of the isoamylase type. When compared with the wild-type plant, mutants lacking isoamylase stored only 10% of the normal level of starch, and instead, accumulated the highly branched water-soluble phytoglycogen. Both normal starch and phytoglycogen accumulated simultaneously in the same chloroplasts in the mutant lines, suggesting to the authors that isoamylase had an indirect rather than a direct role in determining amylopectin structure in *Arabidopsis* [Zeeman, *et al.*, 1998 Vol 5 Tab 81].

Based on a study with *Chlamydomonas*, Ball and co-workers modified the model of amylopectin synthesis described above to a 'discontinuous synthesis' model. According to this theory, "glucan trimming" by DBEs is required to achieve order in the amorphous lamella in order to accommodate synthesis of the crystalline lattice seen in amylopectin molecules [Sun, *et al.*, 1999 Vol 4 Tab 66].

Fujita *et al.* investigated the cDNA structure of isoamylase from the developing endosperm of rice (*Oryza sativa*). The authors stated that isoamylase (EC 3.2.1.68) was purified and confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), via sequential use of Q Sepharose HP anion-exchange chromatography, ammonium sulfate fractionation, and TSKgel G4000SWXL and G3000SWXL gel

filtration chromatography. The purified rice isoamylase was able to debranch glycogen, phytoglycogen and amylopectin but could not attack pullulan. Although the protein exhibited a molecular size of approximately 83 kDa on SDS-PAGE, the apparent size of the native enzyme was approximately 340 and 490 kDa on TSKgel 63000SWXL and G4000SWXL gel filtration chromatograms, respectively. From these data the authors deduced that rice isoamylase exists in a homo-tetramer to homo-hexamer form in developing endosperm. The optimum pH and temperature for isoamylase activity were pH 6.5 to 7.0 and 30° C, respectively. The enzyme activity was completely inhibited by $HgCl_2$ and *p*-chloromercuribenzoate at 1 mM. These results indicated that rice isoamylase possessed properties that were distinct from those reported for bacterial isoamylase [Fujita, *et al.*, 1999 Vol 2 Tab 18 and Figure 1 below].

Complementary-DNA clones for rice endosperm isoamylase were isolated with a polymerase-chain-reaction product as a probe. The probe was generated by primers designed from nucleotides conserved in cDNA for maize sugary-1 isoamylase and a *Pseudomonas amyloderamosa* gene encoding isoamylase. The nucleotide sequence of the longest clone showed a high similarity to that of maize sugary-1 isoamylase, but a lesser similarity to those of *Pseudomonas amyloderamosa* isoamylase [Fujita, *et al.*, 1999 Vol 2 Tab 18].

Gene mapping and phylogenetic analysis of the proteins indicated that isoamylases from maize and rice were more closely related to a number of GlgX gene products from the blue green alga *Synechocystis* and various bacteria than to isoamylases from *Pseudomonas* and *Flavobacterium*. Further, all isoamylases were more closely related to each other than to pullulanases. As a result the authors proposed that GlgX proteins be classified as isoamylase-type debranching enzymes. Their phylogenetic tree showed that all starch- and glycogen-debranching enzymes from the plants and bacteria tested could be classified into two distinct types, an isoamylase-type and a pullulanase-type (See Figure 1

below).

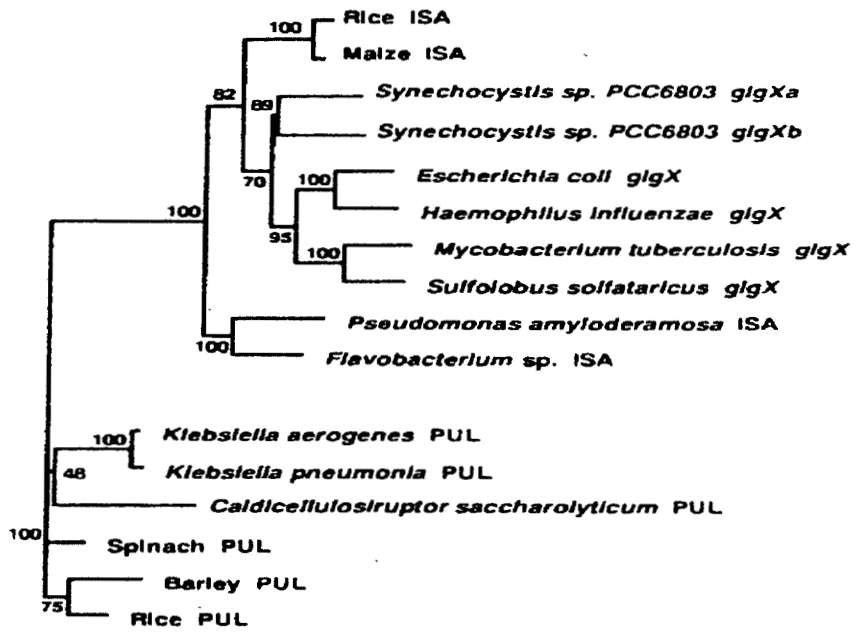


Figure 1
Phylogenetic Analysis of Isoamylase Enzymes
from Fujita, *et al.*, 1999 Vol 2 Tab 18

Sun *et al.* analyzed isoamylase gene activity in wild-type barley, stating that it was as yet unclear whether wild-type plants utilize one, or both debranching enzymes in starch synthesis [Sun, *et al.*, 1999 Vol 4 Tab 66]. A study was designed to determine if isoamylase takes part in starch synthesis by comparing isoamylase gene activity under three conditions: (1) during starch accumulation in developing sink (immature leaves, growing root tips, and developing flowers, fruit, and seed) tissues; (2) during starch degradation in germinating seeds; and (3) in ectopic

expression after applying sucrose, a starch precursor. The investigators isolated the gene for barley isoamylase (iso1), and analyzed its expression and regulation in germinating seeds, developing endosperm and vegetative tissues, and compared the isoamylase gene expression in sink tissues from three different species. Their results linked the activity of the isoamylase DBE gene, iso1, to starch synthesis during endosperm development in wild-type plants [Sun, *et al.*, 1999 Vol 4 Tab 66]. Further, they demonstrated that the iso1 gene is induced by sucrose, a result that is in direct contrast to α-amylases, which are repressed by sucrose [Sun, *et al.*, 1999 Vol 4 Tab 66].

Kubo *et al.* showed that both enzymes, isoamylase and pullulanase were involved in amylopectin biosynthesis in rice (*Oryza sativa*) endosperm [Kubo, *et al.*, 1999 Vol 3 Tab 36]. The authors indicated that pullulanase was either also essential or could compensate for the role of isoamylase in the construction of the amylopectin multiple-cluster structure. They suggested that it was likely that isoamylase was modified in some sugary-1 mutants such as *EM-273* and *EM-5*, since it was present, but was apparently inactive. Their results showed that the sugary-1 gene encodes the isoamylase gene of the rice genome [Kubo, *et al.*, 1999 Vol 3 Tab 36].

Mutants of *Chlamydomonas reinhardtii* were also shown to accumulate glycogen in the absence of an enzyme with isoamylase-type specificity [Dauvillee, *et al.*, 2000 Vol 2 Tab 13]. While investigating the functions of debranching enzymes in starch biosynthesis, the authors partially purified and characterized enzymes from wild type and mutant *sta7 Chlamydomonas reinhardtii.* Mutants of the STA7 locus were found to substitute synthesis of small quantities of a glycogen-like material for synthesis of insoluble granular starch. The mutants were previously shown to lack an 88 kDa debranching enzyme. Two distinct debranching activities were detected in wild-type strains. In glycogen-producing mutants (CIS1) the missing 88 kDa debranching enzyme subunit was shown to be part of a multimeric

enzyme complex. A monomeric 95 kDa debranching enzyme (CLD1) cleaved α-1,6 linkages separated by as few as three glucose residues, while the multimeric complex was incapable of that activity. Both enzymes were able to debranch amylopectin. The α-1,6 linkages of glycogen were only completely debranched by the multimeric complex. Taken together, CLD1 and the multimeric debranching enzyme display both limit-dextrinase (pullulanase) and isoamylase-type specificities. The authors found that various mutations in the STA7 locus caused the loss of both CIS1 and the multimeric isoamylase complex. In contrast to rice and maize mutants that accumulate phytoglycogen when mutations of an isoamylase-type DBE occur, isoamylase depletion in *Chlamydomonas* did not result in any differences in pullulanase activity [Dauvillee, *et al.*, 2000 Vol 2 Tab 13].

b. Substrate Specificity

Carbohydrate-debranching enzymes are generally classified according to their specificities for different substrates [Evans, *et al.*, 1971 Vol 2 Tab 15]. Manners *et al.* proposed a classification based on substrate specificity for the 6 main types of debranching enzymes known to hydrolyze saccharide α-1,6-D-glycosidic, but not α-1,4-D-glucosidic, linkages. These enzymes included amylopectin 6-glucohydrolase, isoamylase, pullulanase, limit dextrinase, oligo-1, 6-glucosidase, and amylo-1, 6-glucosidase [Manners, *et al.*, 1971 Vol 3 Tab 47]. The various enzymes listed above were known to be capable of debranching one or more of the following carbohydrates: amylopectin, glycogen, glycogen phosphorylase limit dextrins, pullulan, α-dextrins, panose, and isomaltose [Manners, *et al.*, 1971 Vol 4 Tab 47].

Yokobayashi *et al.* reported in 1969, that *Pseudomonas* isoamylase was a debranching enzyme that could hydrolyze amylopectin, glycogen, and β-limit dextrins. However, unlike pullulanase this enzyme could not hydrolyze the α-1,6-glucosidic linkages in pullulan [Yokobayashi, *et al.*, 1969 Vol 5, Tab 78]. Instead, the enzyme was reported to be able to access the interior

of the glycogen molecule, and hydrolyze 100% of its α-1,6-glucosidic linkages. Table 1 below shows that *Pseudomonas* isoamylase has very little activity against pullulan (0.5%), while its activity against various sources of glycogen varies from 50 to 110%. It was found to be most effective against amylopectin, exhibiting specific activities of 74 to 124% [Yokobayashi, *et al.*, 1969 Vol 5, Tab 78, Yokobayashi, *et al.*, 1970 Vol 5 Tab 79 and Hayashibara, 2000 unpublished data].

In 1972 Harada *et al.* characterized *Pseudomonas* isoamylase by its actions on amylopectin and glycogen and compared the activity to that of *Aerobacter* pullulanase. The research team studied the debranching actions of a crystalline isoamylase preparation (previously numbered EC 3.2.1.9) derived from *Pseudomonas* SB-15 [Harada *et al.*, 1972 Vol 2 Tab 22]. Its activity was then compared with those of crystalline pullulanase. Analyses of the debranched glucans produced after various periods of incubation suggested that the *Pseudomonas* isoamylase hydrolyzes both inner and outer branching linkages of amylopectin, and all the branching linkages of glycogen. In contrast, the pullulanase hydrolyzed the outer linkages of amylopectin, but was not effective against the inner linkages or glycogen [Harada, *et al.*, 1972 Vol 2 Tab 22].

Table 1 below depicts the relative specific activity of *Pseudomonas* isoamylase as determined by Yokobayashi and co-workers [Yokobayashi, *et al.*, 1969 Vol 5 Tab 78].

Table 1

Activity of *Pseudomonas* Isoamylase

Substrate		Specific Activity (%)
Amylopectin	Potato	100
	Sweet potato	106
	Wheat	111
	Maize	124
	Waxy maize	96
	Glutinous rice	74
Soluble amylopectin	Waxy maize	53
	Glutinous rice	44
Glycogen	Oyster	73
	Rabbit liver	50
Phytoglycogen	Sweet corn	110
Pullulan		0.50

[Yokobayashi, *et al.*, 1969 Vol 5, Tab 78 and Yokobayashi, *et al.*, 1970 Vol 5, Tab 79]

c. Mode of Action

Isoamylase has been shown to hydrolyze both the inner and the outer branching linkages of amylopectin, and the inner branching linkages of glycogen [Harada, *et al.*, 1968 Vol 2 Tab 21 and Yokobayashi, *et al.*, 1970, Vol 5 Tab 79]. Harada *et al.* reported that the mode of action of isoamylase on amylopectin may be endowise whereas on glycogen, it may be exowise [Harada, *et al.*, 1972 Vol 2 Tab 22].

More recently, Nakamura *et al.* and Fujita *et al.* have shown that isoamylase is necessary for amylopectin synthesis in plants [Nakamura, *et al.*; 1996 Vol 3 Tab 49 and Fujita, *et al.*, 1999 Vol 2 Tab 18]. Previously, it was thought that debranching enzymes were only involved in the degradation of amylopectin in storage starch during germination. Fujita *et al.* suggested that isoamylase plays a specific role in amylopectin biosynthesis in rice and maize endosperm, along with starch synthase, and pullulanase. A reduction in the levels of the starch debranching enzymes was

shown to produce the sugary-1 mutant in maize [Fujita, *et al.*, 1999 Vol 2 Tab 18]. While it is not known if isoamylase is present in all higher plants, it has been reported in maize, rice, barley, peas, and potato [Fujita, *et al.*, 1999 Vol 2 Tab 18, Sun, *et al.*, 1999 Vol 4 Tab 66 and Ishizaki, *et al.*, 1983 Vol 3 Tab 30].

The different modes of attack of pullulanase and isoamylase on wheat amylopectin were studied by Atwell *et al.* The action of pullulanase produced a bimodal distribution of amylase chains throughout the degradation of amylopectin. Isoamylase, however, was reported to have quickly reduced the polymer to intermediate fractions, which were in turn further debranched to form a bimodal distribution [Atwell, *et al.*, 1980, Vol 2 Tab 4]. In this study the starch chain distribution was elucidated by gel chromatography [Atwell, *et al.*, 1980, Vol 2 Tab 4].

Kimura *et al.* studied the activity of isoamylase (EC 3.2.1.68) on native and gelatinized granules. In this study the authors defined gelation as the swelling of and hydration of the starch granules that takes place after heating the granule in water for 1 hour at the temperature known to initiate gelation for the specific type of starch. The reaction of isoamylase with 7 types of starch at 37°C for 32 hours showed that different types of starch had different susceptibilities to the enzyme. Two of the 3 starches that were quite resistant to hydrolysis by glucoamylase, i.e., amylomaize-7 and shoti starches, showed the highest reactivity with isoamylase, 11.9 and 11.6%, respectively (See Table 2 below) [Kimura, *et al.*, 1996 Vol 3 Tab 34]. The authors explained that both of these starches were relatively low in molecular weight and had shorter chains than most of the other starches tested. Maize, waxy maize, and barley starches were intermediate in their susceptibility, reaching reactivities of 7.3, 7.8, and 6.2%, respectively. Tapioca and potato starches were the least susceptible to isoamylase (See Table 2 below) [Kimura, *et al.*, 1996 Vol 3 Tab 34].

Table 2
Reaction Of Native Starch Granules With 10 IU/ml Isoamylase
(37 °C For 32 hours)

Starch	% In Supernatant [1,2]	% In Granule [1,2]	% Total reaction [2,3]
Tapioca	61.1	38.9	3.6
Potato	68.2	31.8	3.7
Barley	75.8	24.2	6.2
Maize	43.8	56.2	7.3
Waxymaize	64.1	35.9	7.8
Shoti	47.4	52.6	11.6
Amylomaize-7	56.3	43.7	11.9

[1] Percent in the supernatant and in the granule represents the percent of the product obtained from the total reaction
[2]The data are reported as the means of triplicate measurements and have an accuracy of ± 0.1%.
[3] Percent reaction was determined by measuring the reducing value produced in the supernatant and in the granule after reaction of the granules with isoamylase divided by the reducing value produced by reaction of isoamylase with solubilized starch.

The results of the Kimura *et al.* study showed that *P. amyloderamosa* isoamylase would hydrolyze the α-(1→6) branch linkages in native starch granules to a limited extent (3.6-11.9%), depending on the type of starch. The authors indicated, that there was a variation in the degree of reaction with the different types of starch granules, and that this result was similar to that observed for glucoamylase. They stated, however, that there was no correlation, between the types of starches that were susceptible to glucoamylase and the types of starches that were susceptible to isoamylase [Kimura, *et al.,* 1996 Vol 3 Tab 34]. There was an increase in the percent of hydrolysis after the starches had been gelatinized (See Table 3 below). A previous study done in the authors' laboratory had shown that there was an increase in the percent of hydrolysis of gelatinized starch granules by glucoamylase, and that potato starch had become highly

susceptible to both glucoamylase and isoamylase [Kimura, *et al.*, 1996 Vol 3 Tab 34].

Table 3
Reaction of Gelatinized Starch Granules
with 10 IU/ml Isoamylase at 45 °C for 144 h

Starch	% Reaction of Gelatinized Granules [1,2]	% Reaction of Native Granules [1,2,3]
Potato	56.2	3.7
Amylomaize-7	36.4	11.9
Shoti	21.8	11.6
Waxymaize	36.4	7.8

[1] Percent reaction was determined by measuring the reducing value produced in the supernatant and in the granule after reaction of the granules with isoamylase divided by the reducing value produced by reaction of isoamylase with solubilized starch.
[2] The data are reported as the means of triplicate measurements and have an accuracy of ±0.1%.
[3] The native starch granules are the results of reaction with isoamylase at 37 °C for 32 h.

Based on the data, Kimura *et al.* stated that while both glucoamylase and isoamylase have starch-binding domains that allow them to attack native starch granules, their hydrolytic mechanisms are obviously different [Kimura, *et al.,* 1996 Vol 3 Tab 34]. According to the authors, glucoamylase is an exo-acting enzyme that requires a non-reducing–end glucose residue for hydrolyzing either the α-(1→4) or α-(1→6) glycosidic linkages. Isoamylase, on the other hand, is an endo-acting enzyme that requires the binding of two starch chains to hydrolyze the α-(1→6)-branch linkage. Those chains were described as the starch chain to which the branch chain is attached, and the branch chain itself [Kimura, *et al.,* 1996 Vol 3 Tab 34].

The authors hypothesized that the requirement for binding the two chains may be the bottleneck that prevents a more extensive hydrolysis in the granule by isoamylase, *i.e.,* when compared to

the amount of hydrolysis that has been observed with solubilized starch. The authors believed the limited hydrolysis of native starch to be due to the structure and conformation of chains in the granule [Kimura, *et al.,* 1996 Vol 3 Tab 34]. The conclusion drawn was that the percent of hydrolysis for both native and gelatinized starch granules by isoamylase was undoubtedly dependent on the steric nature of the starch chains around the α-(1→6) branch linkage. The resistance of at least some of the α-(1→6) branch linkages to isoamylase hydrolysis was most likely due to the starch chains being involved in double helices and in intermolecular associations that are part of the crystalline regions in the granule. The authors explained that the crystalline regions would not form the necessary complexes with the active site of isoamylase. The degree of penetration into the granule and amount of hydrolyzed linkages inside the granule was thought remarkable considering the molecular the size of glucoamylase (M_r = 74,000 Da) and isoamylase (M_r = 86,000 Da). [Kimura, *et al.,* 1996 Vol 3 Tab 34].

In comparison Evans *et al.* reported on the action of yeast isoamylase and bacterial pullulanase on amylopectin. The action of purified yeast isoamylase and bacterial pullulanase on amylopectin resulted in the hydrolysis of the outermost interchain linkages with the release of linear maltosaccharides having an average degree of polymerization of approximately 15 D-glucose residues. The authors stated that this type of hydrolytic action distinguishes yeast isoamylase from yeast amylo-(1→6)-glucosidase, which acts by a combination of transferase and glucosidase activities [Evans, *et al.,* 1971 Vol 2 Tab 15].

Kainuma *et al.* examined the detailed structural requirements for various branched oligo- and polysaccharide substrates of *Pseudomonas* isoamylase [Kainuma, *et al.,* 1978 Vol 3 Tab 32]. These were compared with the requirements for *Klebsiella aerogenes* pullulanase. The study showed that as with pullulanase, isoamylase is unable to cleave D-glucosyl stubs from branched saccharides. However, *Pseudomonas* isoamylase was

reported to differ from pullulanase in several characteristics. Isoamylase is most effective with higher-molecular-weight polysaccharides. In this study most of the branched oligosaccharides examined were hydrolyzed at a lower rate (10% or less) than the rate of hydrolysis for amylopectin. Isoamylase was found to cleave maltosyl branches very slowly in comparison with maltotriosyl branches. The investigators concluded that *Pseudomonas* isoamylase requires a minimum of 3 D-glucose residues in the B- or C-chain of the polysaccharide in order to hydrolyze the carbon chains [Kainuma, *et al.,* 1978 Vol 3 Tab 32].

d. Molecular Weight and Structure

A reliable molecular weight of the crystalline form of isoamylase from *P. amyloderamosa* was obtained by SDS-polyacrylamide gel electrophoresis and by gel filtration on Sepharose-613 in 6M guanidine-HCl after heat inactivation in the laboratory [Amemura, *et al.*, 1980 Vol 2 Tab 2]. The molecular weight of the undegraded polypeptide chain of the enzyme was found to be approximately 90,000. The authors stated that the lower molecular weight and the description of the subunit structure of the enzyme as reported previously were incorrect [Amemura, *et al.*, 1980 Vol 2 Tab 2]. More recent reports have shown that the molecular weight of Hayashibara isoamylase is approximately 80,000 Da [Hayashibara, 2000, unpublished data].

For the purpose of understanding the substrate specificity and the catalytic mechanism of *Pseudomonas* isoamylase, Katsuya *et al.* published a study on the three-dimensional structure of isoamylase from *P. amyloderamosa* [Katsuya, *et al.,* 1998 Vol 3 Tab 33]. The authors explained that the three-dimensional structures of the enzymes that catalyze the hydrolysis of α-1,4-glucosidic linkages have been determined, including endo-type α-amylases and exo-type maltotetraose-forming α-amylases. Further, the three-dimensional structures of other α-amylase-related enzymes have also been determined, including cyclodextrin glycosyltransferase from *Bacillus circulans* and

cyclodextrin glycosyltransferase from *Bacillus stearothermophilus*. The authors stated that most of these structures have been further investigated in the presence of bound substrates or substrate analogues [Katsuya, *et al.*, 1998 Vol 3 Tab 33]. According to Katsuya *et al.,* these studies have extended the understanding of the mode of substrate binding and the role of catalytic residues. However, until the publication of their study, the three-dimensional structures of the enzymes that hydrolyze α-1,6-glucosidic linkages had not yet been determined, except for that of oligo-1, 6-glucosidase from *Bacillus cereus*.

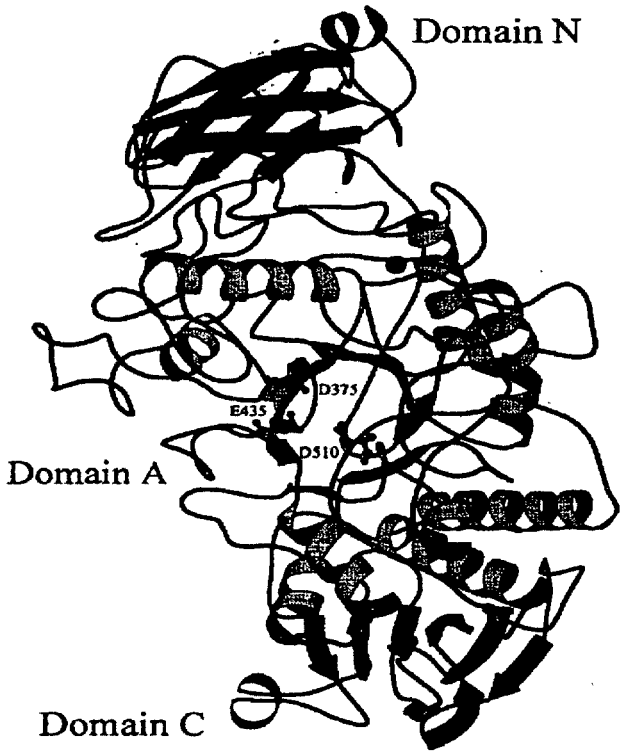
Isoamylase is one member of the α-amylase family of enzymes that includes enzymes such as cyclodextrin glycosyltransferases (CGT), pullulanase, neopullulanase and branching enzymes. The research team determined the structure of the enzyme by X-ray structure analysis at 2.2 Å resolution. The refined structure was described and compared with the structures of related enzymes.

The *P. amyloderamosa* isoamylase was found to be composed of 750 amino acid residues with a molecular weight of 80 kDa [Amemura, *et al.,* 1988 Vol 2 Tab 3 and Chen, *et al.,* 1990 Vol 2 Tab 12]. The amino acid sequence of *Pseudomonas* isoamylase had been previously deduced from the nucleotide sequence of the cloned gene [Amemura, *et al.,* 1988 Vol 2 Tab 3 and Chen, *et al.,* 1990 Vol 2 Tab 12]. Four regions were described that were homologous with those conserved in α-amylases [Katsuya, *et al.,* 1998 Vol 3 Tab 33]. The authors stated that predictions, based on an analysis of amino acid sequence homology, showed that the three-dimensional structure of the core portion of isoamylase was similar to that of α-amylases [Katsuya, *et al.,* 1998 Vol 3 Tab 33].

The investigators elucidated the isoamylase crystal structure by the multiple isomorphous replacement method and refined the structure at 2.2 Å resolution. The analysis revealed that in the N-terminal region, (See Figure 2 below), the enzyme possessed a special domain that was designated "N". The 3-dimensional

structure of this domain had not previously been reported [Katsuya, *et al.,* 1998 Vol 3 Tab 33]. The N domain was reported to have a $(\beta/\alpha)_8$-barrel-type supersecondary structure in the catalytic domain. The authors reported that this type of structure was common to the α-amylase family of enzymes, although in the case of isoamylase, the barrel was incomplete, with a deletion of an α-helix between the 5th and 6th β-strands. Isoamylase was also found to contain bound Ca_2^+ at the interface of domains A and N (See Figure below). All other α-amylase enzymes are known to contain bound Ca_2^+ between domains A and B [Katsuya, *et al.,* 1998 Vol 3 Tab 33]. The authors concluded that their study would advance the understanding of the interactions of isoamylase with its appropriate substrates.

Figure 2
Three-Dimensional Structure of *Pseudomonas* Isoamylase
From Katsuya, *et al.*, 1998 Vol 3 Tab 33



e. Isoelectric Point

Yokobayashi *et al.* found that the isoelectric point of the enzyme was 4.4 using electrophoresis on cellulose polyacetate strips [Yokobayashi, *et al.*, 1970, Vol 5 Tab 79].

f. Kinetic Properties

Yokobayashi *et al.* [Yokobayashi, *et al.*, 1973 Vol 5 Tab 80], studied the kinetic properties of isoamylase. The data are shown below in Table 4. In this study 4 ml of various concentrations of substrate, 0.5ml of 0.1 M acetate buffer (pH 3.5), and 0.5 ml of crystalline enzyme solution containing 0.0134 µg isoamylase (51

μg isoamylase when pullulan was the substrate) were mixed. After a 30-minute period at 40°C, the reducing power of the hydrolysate was analyzed. Based on these results, the authors concluded that isoamylase had a higher affinity and activity for glycogen and for amylopectin, than did pullulanase (data for pullulanase not shown), whereas pullulanase had a higher affinity for pullulan [Yokobayashi, *et al.*, 1973 Vol 5 Tab 80].

Table 4
Michaelis Constants and V Values of *Pseudomonas* Isoamylase on Various Substrates

Substrate		K_m X 10^4 (g/ml)	V_{max} (μ moles/min/mg-protein)
Amylopectin	Potato	0.14	225
	Sweet potato	0.13	240
	Wheat	0.14	250
	Maize	0.13	280
	Waxy maize	0.17	216
	Glutinous rice	0.13	170
Soluble amylopectin	Waxy maize	0.15	120
	Glutinous rice	0.10	100
Glycogen	Oyster	0.11	165
	Rabbit liver	0.12	115
Phytoglycogen	Sweet corn	0.10	240
Pullulan		2.0	1.1

More recently, Liu *et al.* studied isoamylase, which was immobilized on carboxymethyl-cellulose and chitin. The immobilization on chemically modified carboxymethyl-cellulose (CM-cellulose azide) resulted in a specific activity of 1422 U/g-CM-cellulose-isoamylase (CMCI), and a 24% activity retention. The optimal pH was found to be 4.0. The immobilization of isoamylase on glutaraldehyde treated chitin yielded 1638 U/g-chitin-isoamylase (CI), 46% activity retention, at an optimal

pH of 2.4. The kinetic data (Km) indicated that CI (0.69 g/L) had a similar mass transfer resistance when compared to the free enzyme (0.67 g/L), whereas CMCI (3.57 g/L) had a much higher transport resistance [Liu, *et al.*, 1997 Vol 3 Tab 44].

g. Specific Activity

Yokobayashi *et al.* determined the specific activity of *Pseudomonas* isoamylase using a method, which the investigators had published previously [Yokobayashi, *et al.*, 1970, Vol 5 Tab 79]. The specific activity was determined to be 59,100 units (U) per mg of protein from the culture fluid of *Pseudomonas sp.* strain SB-15.

A mutant of strain SB-15, MI-414, which was produced by treating the parent strain with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, was also found to have the same specific activity (~ 59,000 U/mg of protein). However, in the case of strain MI-414, isoamylase production was about 10 times that of strain SB-15 [Fujita, *et al.*, 1999 Vol 4 Tab 29].

Using improved substrains of SB-15, such as MU 1174, Hayashibara Biochemical Company has developed a commercial enzyme product containing greater than or equal to 1.25 $X10^6$ U/g of enzyme solution [Hayashibara, 2000, unpublished data Vol 2 Tab 25]. The specific activity of the enzyme purified to homogeneity has been determined to be 59,1000 U/mg [Hayashibara, 2000, unpublished data Vol 2 Tab 25]. This level of activity is currently set into the commercial specifications for the enzyme.

h. Enzymatic Activity (unit of activity)

One unit of Hayashibara isoamylase activity is defined as the amount of enzyme that is capable of increasing the absorbency of a defined reaction mixture by 0.008 at 610 nm [Hayashibara, 2000, unpublished data]. The company has provided an analytical

method for the determination of activity.

1. Analytical Method for Isoamylase Activity

   A reaction mixture, consisting of 3 ml of 0.833% Lintner soluble waxy corn starch (100mM acetate buffer, pH 3.5), and 0.5 ml of enzyme solution (10mM acetate buffer (pH 4.5) used as diluent) was incubated under agitation at 40°C for 30 minutes. A 0.5 ml aliquot of the reaction mixture was removed at 0.5 minutes and again at 30.5 minutes. These aliquots were mixed with the reaction stop solution consisting of 15 ml of 0.02 N sulfuric acid (stored at 25°C) and 0.5 ml of 0.01 N iodine solution. This solution was allowed to stand at 25°C for 15 minutes. The absorbance of the iodine-starch complex was measured at 610 nm.

2. Definition of Enzyme Activity

   One unit of activity was defined as the amount of enzyme that is capable of increasing absorbance by 0.008 [Hayashibara, 2000, unpublished data].

3. Calculation

   The enzyme activity was calculated in accordance with the following expression [Hayashibara, 2000, unpublished data].

$$\text{Activity (u/g or u/ml)} = (E_{30} - E_0) / 0.008 \times 1 / 0.5 \times f \times (\text{dilution ratio})$$
$$= (E_{30} - E_0) \times 250 \times f \times (\text{dilution ratio})$$

Where:

f = a factor of the substrate. A coefficient used to correct discrepancies among production lots of the substrate. The value is renewed each time the substrate is changed.

E_0 = Absorbency of the blank solution (absorbency obtained at 30 seconds after initiation of reaction)

E_{30} = Absorbency of the test solution (absorbency obtained at 30 minutes and 30 seconds after initiation of reaction)

i. Enzymatic Activity Characterization

Yokobayashi *et al.* published information on the properties of *Pseudomonas* isoamylase [Yokobayashi, *et al.*, 1970 Vol 5, Tab 79]. Table 5 below lists the parameters under which the enzyme shows optimal activity. The enzyme shows optimal activity at temperatures ranging from 50-55°C and at pH levels between 3 and 4. It is only weakly inhibited by common inorganic ions and compounds such as iodoacetate (10 mM showed a 13% inhibition) [Yokobayashi, *et al.*, 1970 Vol 5, Tab 79].

Wu *et al.* studied the effect of pH on isoamylase production by *Pseudomonas amyloderamosa* WU 5315 [Wu, *et al.,* 1994 Vol 5 Tab 77]. The isoamylase activity of *Pseudomonas amyloderamosa* WU 5315 was reported to be stable over a pH range of from 5.5 to 6.25, but only about 30% of the activity remained at pH 6.5. Cells grown at high pH produced what was characterized as low isoamylase activity (418 U/ml). Activity reached nearly 3000 U/ml when a pH level of less than 6.0 was maintained during fermentation. When glucose was substituted for 1/3 of the maltose (3%), which was used as the carbon source, in the fermentation medium, no pH control was required and the isoamylase activity of *P. amyloderamosa* WU 5315 increased to 3400 U/ml [Wu, *et al.,* 1994 Vol 5 Tab 77].

Table 5
Properties of *Pseudomonas* Isoamylase

Temperature

Stability:	Stable at less than 45°C
Optimal activity:	50-55°C

PH

Stability:	Stable at pH 2.5-7.5
Optimal activity:	pH 3-4

Inorganic ions

NaCl (1 mM):	No inhibition
$MgCl_2$ (1 mM):	No inhibition
CuCl (1 mM):	Weak inhibition (30%)
$HgCl_2$ (1 mM):	Weak inhibition (41%)

j. Description of Any Permanent or Transient Structural Modifications Added Through Chemical or Genetic Means e.g., To Improve Stability or Binding To a Support

Not Applicable – Hayashibara isoamylase is not chemically or genetically engineered or altered.

k. Comparison of the Physio-Chemical and Functional Characteristics of a Recombinant Enzyme (If Applicable) with Those of Its Original Counterpart

Not Applicable – Hayashibara isoamylase is not genetically engineered.

6. Composition

a. The nutritional composition of Hayashibara isoamylase is shown in Table 6 below. The mean of triplicate analyses for moisture, ash, carbohydrates and fatty acids is given for lots: 91022, 91119, 00218, 00811, and 01110. The protein level of the commercial grade enzyme was 19 mg per 1g of fluid [Lina, 2000 Vol 3 Tab 43]. Moisture ranged from approximately 56 to 60%, and ash ranged to 0.13 to 0.34%. (Note that enzyme activity is normalized with water) [See Section II Manufacturing Process]. The carbohydrate content of 37.24% originated from the crystalline sugars used as the stabilizer system (See section b below). Likewise, a 0.1% portion of fatty acids was also an ingredient in the stabilizer system. The Table shows that from 0.64 to 4.69% of the enzyme preparation is composed of non-protein organics derived from the enzyme solution [Hayashibara, 2000, unpublished data Vol 2 Tab 25].

Table 6

Nutritional Profile of Hayashibara Isoamylase

Lot No. Mean of Triplicate Analyses	Moisture (%)	Ash (%)	Carbohydrate (%)	Fatty Acids (%)	Protein[1] (%)	Total (%)	Remaining Non-Protein Organics (%)
91022	58.82	0.23	37.24	0.1	1.9	98.29	1.71
91119	58.60	0.13	37.24	0.1	1.9	97.97	2.03
00218	58.79	0.32	37.24	0.1	1.9	98.35	1.65
00811	55.73	0.34	37.24	0.1	1.9	95.31	4.69
01110	59.92	0.20	37.24	0.1	1.9	99.36	0.64

[1] from Lina, 2000, as reported by TNO Laboratories.

b. The Hayashibara isoamylase preparation contains certain ingredients, which serve to stabilize the enzyme preparation. The composition of the stabilizer system is shown in Table 7 below. The stabilizer system comprises 37.34% of the enzyme preparation on a dry weight basis

[Hayashibara, 2000 unpublished data Vol 2 Tab 25]. The principal stabilizers are crystalline glucose and crystalline maltose, each of which is formulated at 20% (as is basis) of the preparation. A fatty acid mixture is added as an emulsifier at 0.10% [Hayashibara, 2000 unpublished data Vol 2 Tab 25].

Table 7
Hayashibara Isoamylase Stabilizer System

Ingredients	(%)	Dry Solids (%)	Dry Matter (%)
Crystalline Glucose	20.0	91.5	18.30
Crystalline Maltose	20.0	94.7	18.94
Fatty Acid/Glycerol	0.10	100	0.10
Total Stabilizer System			37.34

c. The protein content of the Hayashibara isoamylase enzyme preparation has been reported most recently by TNO Laboratories, The Netherlands. The protein concentration was 19 mg/g liquid enzyme preparation [Lina, 2000 Vol 3 Tab 43].

d. Yokobayashi *et al.* reported that the enzyme preparation yielded a single band associated with isoamylase activity following electrophoresis on acrylamide gel (pH 8.3). Additionally, ultracentrifugation of an identical sample yielded a single symmetrical schlieren peak [Yokobayashi, *et al.*, 1970 Vol 5 Tab 79]. The authors determined, based on the data above, that the isoamylase preparation was free of α-amylase, maltase, isomaltase, pullulanase, dextranase, and D-enzyme (forms glucose from maltotriose) activities.

e. The Total Organic Solids (TOS) content of the commercial Hayashibara preparation is shown in Table 8 (below) for lot numbers: 91022, 91119, 00218, 00811, and 01110. The mean TOS for the 5 lots tested (based on triplicate analyses) ranged from 2.54 to 6.59%. The mean of means was determined to be

4.05% [Hayashibara, 2000, unpublished data Vol 2 Tab 25].

Table 8
Hayashibara Isoamylase Preparation
Total Organic Solids (TOS)

Lot No. Mean of Triplicate Analyses	Moisture[1] (%)	Ash (%)	Carbohydrate (%)	Fatty Acids (%)	Total Organic Solids (%)
91022	58.82	0.23	37.24	0.1	3.62
91119	58.60	0.13	37.24	0.1	3.93
00218	58.79	0.32	37.24	0.1	3.55
00811	55.73	0.34	37.24	0.1	6.59
01110	59.92	0.20	37.24	0.1	2.54

[1] Note that enzyme activity is normalized with water [See Section II].

Section II. Manufacturing Process

1. Genetic and Taxonomic Identification of the Source Organism

Pseudomonas amyloderamosa

The parent strain of the enzyme-producing organism has been deposited in the American Type Culture Collection (ATCC), and an Accession Number has been assigned: ATCC 21262 [Pienta, *et al.*, 1996 Vol 4 Tab 55]. The taxonomy of the organism is shown in the lists below [Hayashibara, 2000 unpublished data].

The parent strain was isolated from soil by Harada *et al.* in 1968, and was identified as belonging to the genus *Pseudomonas*. The organism was described as a non-sporeforming, gram-negative rod, which was motile by means of a single polar flagellum. At the time, the strain was believed to be a new species [Harada, *et al.*, 1968 Vol 2 Tab 21]. The strain was later identified as *amyloderamosa*.

Taxonomy

Kingdom:	*Procaryotae*
Family:	*Pseudomonadaceae*
Genus:	*Pseudomonas*
Species:	*amyloderamosa*

Taxonomic Characteristics

Gram Reaction:	Negative
Cell Shape:	Rod
Cell Diameter:	0.4-0.6 μm
Spore Forming:	Negative
Flagellar Arrangement:	Polar, Single
Growth:	Aerobic
Optimum Temperature	25-30° C
No growth at:	37° C
Optimum pH:	6.5-7.5
Ammonia Production:	Positive
H_2S Production:	Negative
Indole Production:	Negative
Gelatin Utilization:	Positive
Catalase Production:	Positive
Urease Production:	Positive
Methyl Red Stain:	Negative
Nitrate Reduction:	Negative

Table 9 below depicts the sugars and other substrates utilized by *Pseudomonas amyloderamosa.*

Table 9
Utilization of Sugars and Other Carbohydrates

Substrate	Utilization	Acid Production
Arabinose	+	+
Glucose	+	+
Mannose	+	+
Fructose	+	-
Galactose	+	-
Lactose	+	-
Cellobiose	+	-
Trehalose	+	-
Salicin	+	-
Methyl-glucoside	+	-
Xylose	-	NT[1]
Rhamnose	-	NT
Sorbitol	-	NT
Mannitol	-	NT
Inositol	-	NT
Glycerol	-	NT
Benzoic Acid	-	NT
Salicyclic Acid	-	NT
Citric Acid	-	NT
Succinic Acid	-	NT

[1]NT =not tested

2. Natural History of the Enzyme

Norrman *et al.* published a review of the comparative biochemistry of α-glucan-utilization in *Pseudomonas amyloderamosa* and *Pseudomonas saccharophila*. The authors also provided an explanation of the physiological significance of variations in the pathways [Norrman, *et al.*, 1975 Vol 4 Tab 51]. The authors found that maltose, maltodextrins, and glycogen supported excellent growth of both organisms and were extensively metabolized. *P. amyloderamosa* produced limited growth on amylopectin and the

carbohydrate was only partly degraded. The explanation given was that it seemed likely that many of the unit chains liberated from amylopectin had a length exceeding the substrate range accepted by the maltodextrin permease system. A correlation was established between the pH of the medium and the utilization of glycogen and amylopectin for growth in *P. amyloderamosa*. A lack of growth at pH 6.5 was thought to reflect the low activity of isoamylase at this pH. Intracellularly, maltodextrin phosphorylase and 4-α-glucanotransferase occurred in both bacteria. Degradation of extracellular α-glucans was reported to be mediated by an isoamylase transported to the external environment by *P. amyloderamosa*, whereas *P. saccharophila* possessed an extracellular α-amylase and a firmly cell-bound pullulanase [Norrman, *et al.,* 1975 Vol 4 Tab 51].

Norrman *et al.* provided two schematics to depict the metabolism of α-glucans by native *Pseudomonads*. Figure 3 below shows the similarities and differences in enzyme patterns used by 3 *Pseudomonas* species to metabolize α-glucans [Norrman, *et al.,* 1975 Vol 4 Tab 51]. Figure 4 illustrates the general scheme for moving maltodextrins into the cell [Norrman, *et al.,* 1975 Vol 4 Tab 51]. In Figure 4 either isoamylase or pullulanase could be utilized to perform debranching functions.

Figure 3
Enzyme Patterns of Three *Pseudomonas sp.*

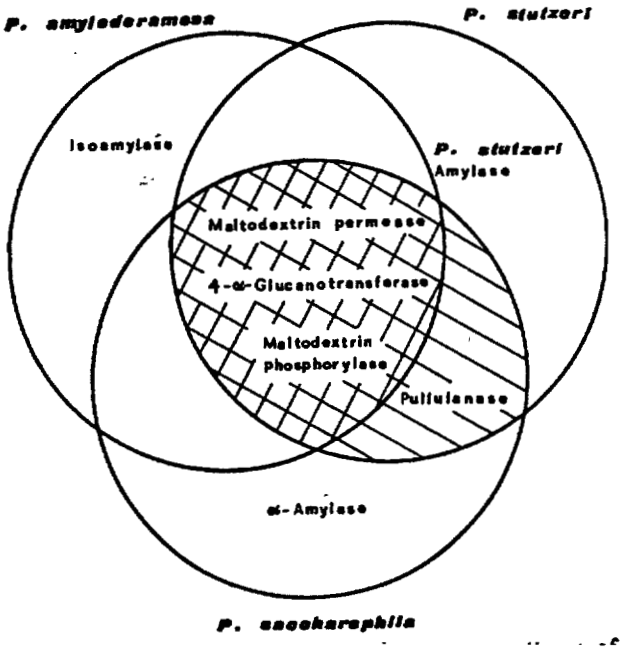
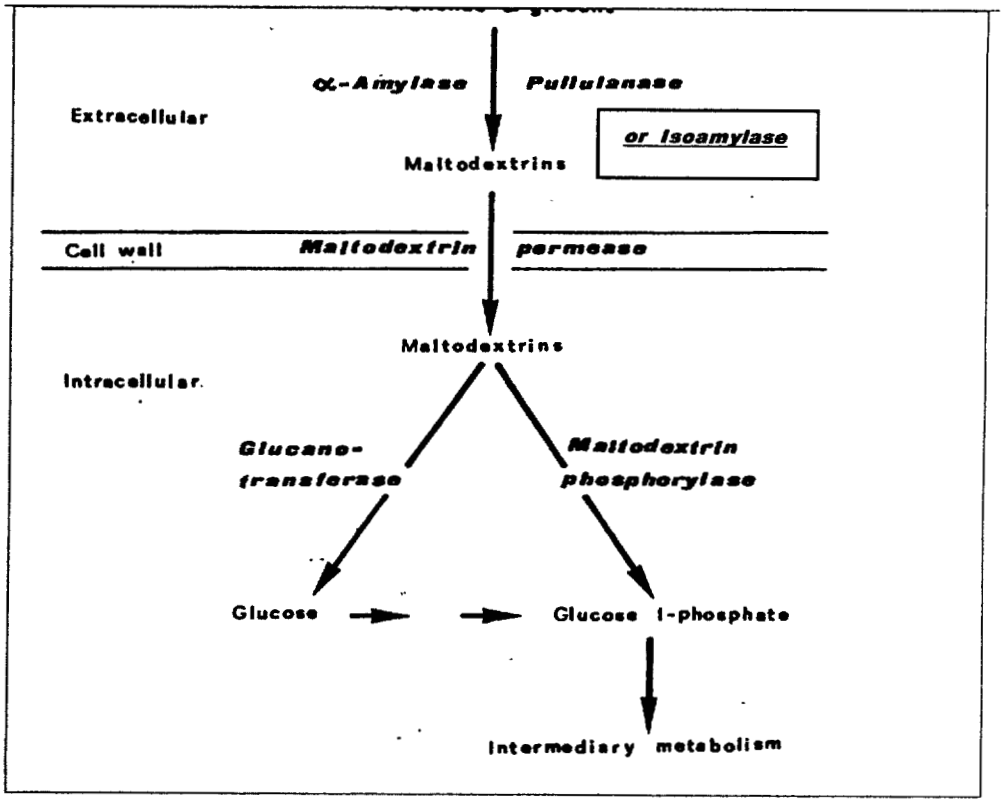


Figure 4
General Scheme for Transporting Maltodextrins through the Cell Wall



Sugimoto *et al.* found that *Pseudomonas* SB-15 and a mutant strain, produced an extracellular isoamylase, and an intracellular α-glucosidase and amylase(s) when grown on maltose. The mutant strain (*MS1*) derived from SB-15, formed isoamylase as part of its normal enzyme production, along with intracellular α-glucosidase and amylase(s) [Sugimoto, *et al.*, 1974 Vol 4 Tab 65]. The activities of the enzymes produced by the mutant strain were reported to be much higher than those induced in the parent strain.

Harada *et al.* reviewed the production of extracellular isoamylase and intracellular specific α-amylase(s) produced by *Pseudomonas amyloderamosa*. The review also covered the production of the typical α-glucosidase by the same species [Harada, *et al.*, 1977 Vol 2 Tab 23].

3. Isoamylase Production by Other Naturally Occurring Organisms

Other naturally occurring organisms have also been reported to produce isoamylase. Isoamylase obtained from three different genera of bacteria (*Bacillus*, *Flavobacterium* and *Pseudomonas*) is approved in Japan as a food additive [Japanese Food Additive Regulations , 1998 Vol 3 Tab 31]. These have been used for several years to produce various foods and food products. The isoamylase produced from *Pseudomonas amyloderamosa* has been approved in Japan for approximately 20 years and over 300,000 kg have been used to produce both food and pharmaceutical products.

Hizukuri *et al.* reported on the properties of *Flavobacterium odoratum* isoamylase purified from the culture filtrate [Hizukuri *et al.*, 1996 Vol 3 Tab 28]. The molecular weight and isoelectric point of the enzyme were 78,000 and 8.7, respectively. The optimum temperatures for activity were 50 and 45°C in the presence and absence of $CaCl_2$ (1 mM) respectively, and the optimum pH was 6.0 in the presence of $CaCl_2$ (1 mM). The enzyme was reported to completely debranch amylopectin and glycogen, but pullulan remained virtually unhydrolyzed [Hizukuri, *et al.*, 1996 Vol 3 Tab 28].

Likewise an isoamylase with a neutral pH optimum from a *Flavobacterium* species was characterized by Krohn *et al.* [Krohn, *et al.,* 1997 Vol 3 Tab 35]. Active isoamylase was expressed from a 4.9-kb pst1 fragment in *Escherichia coli*, and was detected in the extracellular medium by a plate assay. The isoamylase was 778 amino acids in size [Krohn, *et al.*, 1997 Vol 3 Tab 35]. The amino acid sequence of this isoamylase contained an N-terminal signal peptide of 32 amino acids, and was 61% homologous with *Pseudomonas amyloderamosa* isoamylase. The mature enzyme, which was purified to homogeneity, had a relative molecular mass of 83 kDa, a pH optimum of 6-7, and cleaved glycogen, but did not cleave pullulan. [Krohn, *et al.,* 1997 Vol 3 Tab 35].

Abe *et al.* cloned the isoamylase gene from *Flavobacterium odoratum* KU into *Escherichia coli* [Abe, *et al.,* 1999 Vol 2 Tab 1]. The enzyme produced was excreted into the culture medium, in proportion to the amount of enzyme expressed. The enzyme found in the culture medium had nearly identical characteristics including molecular weight, and the N-terminal sequence as those of the enzyme accumulated in the periplasmic space. This result indicated that the enzyme accumulated in an active form at the periplasm and was transported out of the cell. The primary sequence showed that the mature enzyme consisted of 741 amino acid residues. The researchers found that Asp-374, Glu-422, and Asp-497 were essential, and that the sequences around those residues were highly conserved in isoamylases of different origins [Abe, *et al.,* 1999 Vol 2 Tab 1].

An isoamylase, or glycogen 6-glucanohydrolase (EC 3.2.1.68), was isolated from *B. amyloliquefaciens* by Urlaub *et al.* [Urlaub, *et al.,* 1975 Vol 4 TAB 75]. The enzyme preparation was inactive towards pullulan, maltodextrins, and maltose. Glycogen incubated consecutively with the *Bacillus* isoamylase and β-amylase was converted quantitatively to maltose, indicating complete debranching. The report concluded that *B. amyloliquefaciens* appears to produce an isoamylase under appropriate nutritional conditions.

In a second study by the same research team, releasable α-amylase and isoamylase and membrane-bound α-glucosidase activities were detected in *B. amyloliquefaciens* ATCC 23350, but pullulanase, 4-α-glucanotransferase, and α-glucan phosphorylase activities were not found. A scheme for the metabolism of branched α-glucans was proposed based on the distribution of subcellular enzymes (See Figure 4 above) [Urlaub, *et al.*, 1975 Vol 4 TAB 75].

An extracellular isoamylase from *Flavobacterium* species was purified [Sato, *et al.*, 1980 Vol 4 TAB 58]. The debranching activity of the purified enzyme was detected as a single band by polyacrylamide gel electrophoresis. The enzyme efficiently hydrolyzed α-1,6-glucosidic linkages of glycogen and amylopectin and formed amylose chains, but did not hydrolyze pullulan. The authors indicated that the action of the isoamylase was similar to other microbial isoamylases, although its physical properties were different.

In another study isoamylase was prepared by affinity chromatography of a commercial enzyme preparation obtained from a strain of *Cytophaga* (also described as a *Flavobacterium* or *Polyangium*). The isoamylase, as isolated, was described as unstable, but the authors found that with the addition of Ca_2^+, stability could be improved. The enzyme preparation had a very low, but significant activity on pullulan and on α-dextrins having maltosyl side chains. This observation was inconsistent with previous reports of the time. The authors hypothesized that this was related to the specificity of isoamylase and other bacterial debranching enzymes [Evans, *et al.*, 1979 Vol 2 TAB 16].

4. Source and Taxonomic Identification of Any DNA Introduced from Other Sources

   No other DNA has been introduced into the isoamylase-producing organism.

5. Steps and Procedures Involved in the Construction (If Any) of the Production Microorganism

   The source organism, *Pseudomonas amyloderamosa* has not been genetically engineered.

6. Description of the Fermentation Process of *Pseudomonas amyloderamosa* with Conditions, Reagents, and Equipment

   a. Steps and Controls Used to Maintain the Proper Growth Conditions, Cultural Purity, and Genetic Stability of *Pseudomonas amyloderamosa*

The production controls for Hayashibara isoamylase are listed below. The company manufactures Hayashibara isoamylase under current Good Manufacturing Practices (cGMP), which are standard for the industry.

Pseudomonas amyloderamosa
Growth Conditions and Controls

Condition/ Step	Control
Temp:	computerized
pH:	computerized (analyzed every 8 hours)

Slant Culture

Cultivated at 28°C for 48 hrs.

Ingredients:
Agar

[redacted]

1st Cultivation (flask)

Cultivated at [redacted]

Ingredients:

Agar

[redacted]

Volume: [redacted]

Process control: Cultures are examined microscopically for contamination

2nd Seed Culture (seed tank)

Cultivated at 28°C for 24 hrs.

Ingredients:

Agar

[redacted]

Volume: [redacted]

Process control: Cultures are examined microscopically for contamination

Main Cultivation (main cultivation tank)

Cultivated at 28°C for 120 hrs.

Ingredients:

Agar

[redacted]

[redacted]

Volume: [redacted]

Process control: Growth rate, pH, and residual sugars are monitored and compared with standard curves

Cultural purity

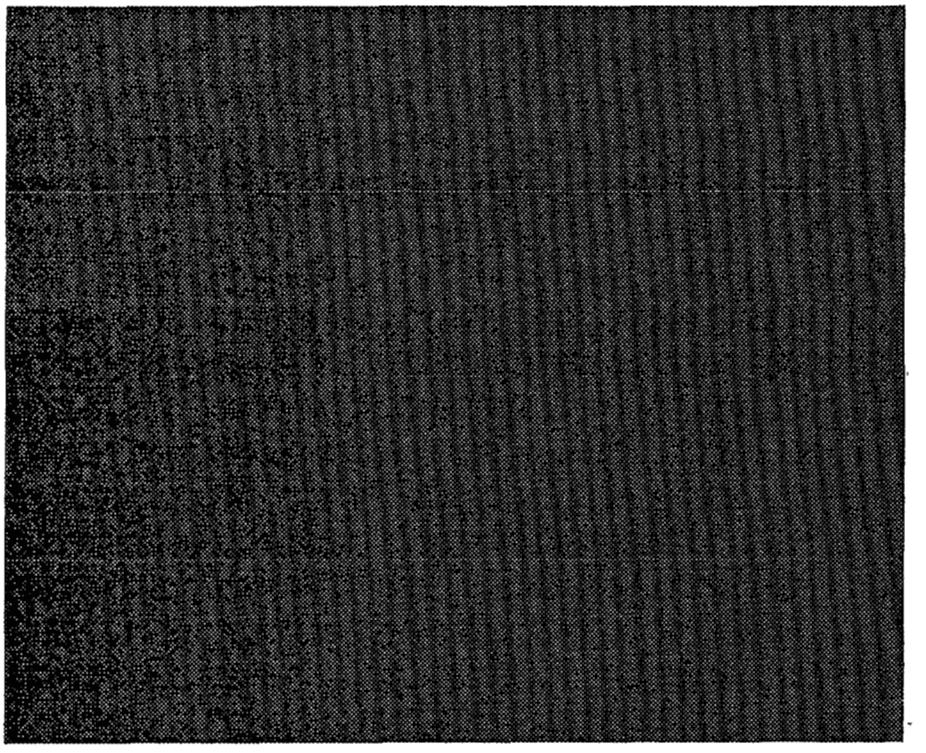
After sterilizing the culture medium at 121°C for 20 minutes, the pressure in the cultivation tank is maintained with sterilized air. After inoculating the seed, the pressure is again maintained with sterilized air. Cultures are routinely examined microscopically to detect bacterial contamination.

For long-term storage, the strain is freeze-dried under vacuum (-15°C). At the current time, Hayashibara does not analyze for genetic stability.

b. Identification of The Components of The Fermentation Medium

Components of the fermentation medium are listed below. Each ingredient is purchased under cGMP according to the company's raw material specifications.

Agar



Water

c. Description of the Isolation of the Enzyme from the Cellular Material or from the Growth Medium

The isoamylase enzyme is not isolated from the growth medium in the Hayashibara process. Rather the source microorganism is separated from the medium via membrane filtration. The purification steps for isoamylase are listed below along with their respective critical controls.

Separation Steps and Controls

Separation (membrane filter)

P. amyloderamosa cells are removed.

Conditions:	Pore size	[redacted]
	Volume of crude broth	[redacted]
	Total area of MF membrane	[redacted]
	Filtration time	[redacted]
	Average flow rate	[redacted]
Process controls:	Temperature, pH, circulating flow rate, and pressure	

Concentration (ultra filter)

Conditions:	Cut-off molecular weight	[redacted] Daltons
	Volume of crude solution (permeate from MF)	[redacted]
	Total area of UF membrane	[redacted]
	Filtration time	[redacted]
	Final concentrated filtrate	[redacted]
	Average flow rate	[redacted]
Process controls:	Temperature, pH, circulating flow rate, and pressure	

Collection of Crude Enzyme

A concentrated solution from the ultra filtration is collected.

Collected volume	[redacted]
Enzyme yield	[redacted]

d. Description of the Procedures for Enzyme Isolation and Purification

After separating the microorganism via membrane filtration, the enzyme solution is concentrated with ultrafiltration, followed by purification with a precoat filter. The solution is then subjected to further membrane filtration in a final purification step.

Filtration (pre-coat filter)

Diatomaceous earth (6 kg) is added for pre-coat filtration.

Conditions:	Volume of solution	[redacted]
	Total area of UF membrane	[redacted]
	Filtration time	[redacted]
	Average flow rate	[redacted]

Filtration (membrane filter)

Conditions:	Pore size	[redacted]
	Pressure	[redacted]
	Average flow rate	[redacted]

Enzyme Preparation (mixing tank)

Stabilizers:	Glucose	20%
	Maltose	20%
	Glycerol ester of fatty acids	0.1%

Adjustment of enzyme activity

Water is added to normalize enzyme activity.

Hayashibara Isoamylase Preparation

Process controls: Compliance with internal specifications is confirmed by testing: enzyme activity, pH, total heavy metals, arsenic, and lead

e. Characterization of Any Immobilizing Agent

Not Applicable - No immobilization agent is used in the Hayashibara isoamylase process.

f. Method of Immobilization (If Applicable)

Not Applicable

Section III. Purity and Food-Grade Specifications

1. Compliance with Food Chemicals Codex specifications for enzyme preparations

Isoamylase meets specifications for enzyme preparations set out in the Food Chemicals Codex [Food Chemicals Codex, 1996 Vol 5 Tab 82]. It is a microbially derived carbohydrase that meets the general requirements of enzymes used in food processing including:

a. Hayashibara isoamylase is produced in accordance with good manufacturing practices (See Manufacturing Section above)
b. Hayashibara isoamylase causes no increase in the total microbial count in the treated food. All cells are removed according to the procedure for separation described in Section II, 4 above, which results in a sterile product.
c. Hayashibara isoamylase is produced using practices designed to produce a controlled fermentation as described in Section II above.
d. Hayashibara isoamylase is not a source of toxins or other undesirable substances, as evidenced by several animal toxicity studies discussed in Section III below.
e. The commercial specifications for Hayashibara isoamylase meet the additional requirements for chemical purity set out in the Food Chemicals Codex.

Assay	Not less than 85% and not more than 115% of the declared activity.
Arsenic (as As)	Not more than 3 ppm
Coliforms	Not more than 30 per g
Heavy Metals (as Pb)	Not more than 0.004%
Lead	Not more than 10 ppm
Salmonella sp.	Negative by test

Isoamylase also meets a list of additional requirements as shown in Table 17 below.

000091

2. Safety and Toxicology Studies

A number of toxicological studies in animals, a bacterial reverse mutation assay, and an *in vitro* mammalian chromosome aberration test in cultured human lymphocytes establish the safety of Hayashibara isoamylase. These studies are discussed below.

a. Acute Oral Pathogenicity and Toxicity Tests in Mice

Morimoto *et al.* conducted a series of three acute tests to assess the toxicity of a preparation of *Pseudomonas amyloderamosa*, and two preparations of the isoamylase enzyme [Morimoto, *et al.*, 1979 Vol 3 Tab 48]. In the first experiment an acute pathogenicity test was performed in male and female mice to using *Pseudomonas amyloderamosa* (wet cells).

Forty (40) female and 40 male ddy-N strain mice, about five weeks old and weighing 20-26 grams, were assigned to four treatment groups per sex. The test sample containing washed bacterial cells was kept frozen and thawed immediately before use. The doses were administered by oral infusion using a gastroprobe [Morimoto, *et al.*, 1979 Vol 3 Tab 48]. Preliminary tests were used to establish the final doses used in the study. The four treatment groups were given doses ranging from 38,200 mg/kg (34.73 ml/kg) to 66,000 mg/kg (60 ml/kg) of wet bacterial cells. Animals were observed for 7 days following treatment.

No mortalities were noted for either sex at any treatment dose. The LD_{50} (oral) value for the test sample was concluded to be greater than 66,000 mg/kg for both male and female mice. This dose was presumed by the investigators to be the highest that could possibly be administered to mice by oral infusion [Morimoto, *et al.*, 1979 Vol 3 Tab 48]. No abnormalities were observed in the general physical condition of mice after administration of the respective doses. The mice started consuming their diet and water within 10-30 minutes after administration, and all groups showed normal growth.

In a second study, the culture filtrate containing isoamylase from *Pseudomonas amyloderamosa* was administered to four groups each of male and female ddy-N strain mice. Each group consisted of 10 five week old mice weighing 18-26 grams [Morimoto, *et al.*, 1979 Vol 3 Tab 48].

The culture filtrate was thawed at room temperature, and a single dose of the solution was administered immediately to the mice by oral infusion using a gastroprobe. A pilot study was used to calculate the 4 doses chosen for this study. The four groups of mice from each sex were administered doses ranging from 34,730 mg/kg (34.73 ml/kg) to 60,000 mg/kg (60 ml/kg). Mice were observed for 7 days following treatment.

No male or female mice at any dosage died subsequent to treatment [Morimoto, *et al.*, 1979 Vol 3 Tab 48]. No abnormalities were observed in the general physical condition of the mice after administration of the respective doses of the *Pseudomonas amyloderamosa* culture filtrate. The observation was made that the mice started consuming their diet and water within 10-30 minutes after the administration. The mice had subsequent normal growth. The investigators concluded that the LD_{50} was greater than 60,000 mg/kg for both male and female mice [Morimoto, *et al.*, 1979 Vol 3 Tab 48].

A second acute toxicity test was performed to assess the effect, in mice, of a concentrated isoamylase preparation from *Pseudomonas amyloderamosa*. In this study ten groups of ddy-N strain mice of both sexes, each group consisting of ten mice about five weeks old and weighing 20-26 grams, were administered a single dose of a 40% aqueous suspension of the test sample by oral infusion using a gastroprobe [Morimoto, *et al.*, 1979 Vol 3 Tab 48]. The authors noted that the test sample tended to appear muddy and to precipitate when allowed to stand. Therefore, the suspension was administered with a rat gastroprobe, while agitating the suspension with a stirrer.

Preliminary results showed that the LD_{50} value for the test sample was approximately 15,000 mg/kg. The test sample was administered at seven doses ranging from 12,000 mg/kg to 21,260 mg/kg. Animals were observed for 7 days following treatment.

All test mice in all dosage groups exhibited bradypragia (sluggishness) and tachypnea (rapid breathing) 10-20 minutes following administration. These clinical signs of most of the surviving mice started to ameliorate 0.5 to 1.0 hour after administration, and the mice appeared normal approximately 2-3 hours after treatment. Conversely, mice that subsequently died lay essentially motionless on their stomach. Shortly before death the animals experienced convulsions, expiring within 1.5 to 15 hours after administration of the suspension. Necropsies of the mice dying from the treatment revealed that the intestinal tracts were turgid containing both the test sample and serofluid. No other abnormalities were noted macroscopically. Additionally, no abnormalities of the intestinal tract were noted in surviving mice at necropsy. Isoamylase was found in the feces 3-5 hours after administration. [Morimoto, *et al.*, 1979 Vol 3 Tab 48].

No deaths occurred in either female or males mice receiving up to 13,200 mg/kg of the enzyme preparation. The LD_{50} (oral) values for the female and males mice were 17,131 mg/kg and 16,908 mg/kg, respectively [Morimoto, *et al.*, 1979 Vol 3 Tab 48].

b. Acute Intravenous Study in Mice

A study was conducted to evaluate the pathogenicity of *Pseudomonas amyloderamosa* administered as a single intravenous injection to mice [Ribera, 2000 Vol 4 Tab 57]. The test material contained various concentrations of *Pseudomonas amyloderamosa* suspended in a 0.9% solution of NaCl. Male and female NMRI Han mice, approximately 5 weeks of age, were

randomly assigned to 4 groups of 10 (5 per sex). Each test animal received a single intravenous injection of 5ml/kg of the microbial preparation. The injections were administered at the rate of approximately 0.020 ml/second. Animals received either the saline control or the bacteria at fixed doses of 4.35×10^{10}, 4.35×10^{9}, or 4.35×10^{8} CFU/kg. Male and female mice were observed for 14 and 15 days, respectively, before study termination. No mortalities or clinical signs were observed in any of the dosage groups, and no significant lesions were recorded upon microscopic examination of the liver, brain, and kidneys of the control or high dose groups [Ribera, 2000 Vol 4 Tab 57]. Further, no viable microorganisms were observed in any of the organs examined.

c. Sub-chronic 13-Week Oral Toxicity Study in Rats

The safety of Hayashibara isoamylase was examined in a sub-chronic 13-week oral toxicity study in male and female Wistar albino rats (Crl:(WI) WU BR) [Lina, 2000 Vol 3 Tab 43]. Body weights of the males ranged from 74 g to 96 g (85 g ± 0.6), while females ranged from 66 g to 84 g (75 g ± 0.6). Rats were divided into four groups of 20 male and 20 female, each. An isoamylase test solution was administered via gavage at doses of 2.5, 5 or 10 ml/kg body weight/day (ml/kg bw/day). The sample used was yellowish-brown and contained 19 mg protein/g of solution, with an activity of 1,362,500 U/g of solution. The solution was kept under refrigeration. The control solution contained the same types and amounts of substances as the treatment, but did not include the enzyme [Lina, 2000 Vol 3 Tab 43].

The treatment doses corresponded to 57, 114, and 228 mg protein/kg bw/day, and to 4,087,500, 8,175,000 and 16,350,000 U enzyme activity/kg bw/day, respectively. In the low and mid dose groups the total volume was adjusted to 10 ml/kg bw with control solution. The control group was given 10 ml of control solution/kg bw/day.

Examination of the study data showed that daily treatment with the isoamylase enzyme preparation for a period of 13 weeks did not cause any adverse effects, even at the highest dose (10 ml/kg bw/day). Therefore, the no-observed-adverse-effect level (NOAEL) of the test substance was placed at the highest-dose level tested, 10 ml/kg bw/day. This dose corresponded to 228 mg of protein/kg bw/day and 16,350,000 U enzyme activity/kg bw/day, or a total treatment period dose of approximately 20.75 g of protein and 14.9 x 10^9 U of Isoamylase [Lina, 2000 Vol 3 Tab 43].

Mean body weights did not show statistically significant differences between the test groups and the controls at any stage of the study. There were no treatment-related changes in food intake or in food conversion efficiency. There were no treatment-related changes in water consumption between the test groups and the controls [Lina, 2000 Vol 3 Tab 43].

The investigator stated that the only statistically significant finding observed in the test groups was an increase in hemoglobin concentration in females receiving the low-and high-doses [Lina, 2000 Vol 3 Tab 43]. The changes were not dose-related, nor were they accompanied by any effects on other red blood cell variables. Hemoglobin concentrations remained within the range of historical control data. Therefore, the findings were considered incidental.

No treatment-related changes in clinical chemistry or other blood variables were observed. Red blood cell variables, clotting variables, and total and differential white blood cell counts were

not significantly different from the controls. No changes in volume and density of the urine were noted. Likewise, there were no differences among the groups in the semi-quantitative observations of the urine or in the microscopy of the urinary sediment. No treatment-related changes were observed in absolute or relative organ weights [Lina, 2000 Vol 3 Tab 43].

The general condition and behavior of the animals were not adversely affected in any of the groups, and no treatment-related changes were noted during an ophthalmic examination [Lina, 2000 Vol 3 Tab 43].

One male of the low-dose group, two females of the mid-dose group and two males and one female of the high-dose group died during the study. The death of these animals was attributed to gavaging failure. The cause of death of two other rats could not be established by the investigators because of cannibalism or autolysis.

The conclusion of the study was that the highest dose of isoamylase did not cause any treatment related adverse affects at the doses tested. The NOAEL was estimated to be 10 ml of test solution/kg bw/day, which is equal to 228 mg of protein/kg bw/day, or 16,350,000 U enzyme activity/kg bw/day.

3. Bacterial Reverse Mutation Test

Hayashibara isoamylase was examined for mutagenic activity in a standardized bacterial reverse mutation assay using the histidine-requiring *Salmonella typhimurium* strains TA 1535, TA 1537, TA 98 and TA 100, the tryptophan-requiring *Escherichia coli* strain *WP2 uvrA*, and a liver fraction of Aroclor-induced rats for metabolic activation (S9-mix) [Van Delft, 1999 Vol 5 Tab 76]. Four bacterial reverse mutation assays were performed; however, the first two studies used a sample concentration much lower than the standard amount and were therefore rerun. Five different concentrations of the test substance were examined in both the absence and the presence

of the metabolic activation system. The test substance concentrations in the final two assays ranged from 62 to 5000 µg of protein per plate. A negative control (water) and positive controls were run simultaneously with the test substance. The concentration and activity of the isoamylase was supplied by the sponsor. Final concentrations were formulated by a dilution method.

The test substance used contained Hayashibara isoamylase produced from cultures of *Pseudomonas amyloderamosa* [Van Delft, 1999 Vol 5 Tab 76]. The protein level of the enzyme solution was 19 mg/gram solution, and the molecular weight of the enzyme is approximately 80,000 Daltons. For this study the test solution did not contain stabilizers. The S9 metabolic activator contained 4mM nicotinamide adenine dinucleotide phosphate, disodium salt and 5mM D-glucose-6-phosphate disodium salt.

The mutagenicity assay was carried out according to the following procedure: 0.1 ml of the appropriate *Salmonella typhimurium* or *Escherichia coli* strains, 0.263 ml of the test substance solution or of the negative control or 0.1 ml of the positive control substance, and 0.5 ml of the S9 mix for metabolic activation or 0.5 ml sodium phosphate 100 mM (pH 7.4) for the non metabolic activation portion of the experiment were added to 2 ml molten top agar (maintained at 46°C). The ingredients were thoroughly mixed with the top agar, and were immediately poured onto minimal glucose agar plates [Van Delft, 1999 Vol 5 Tab 76]. All the determinations were made in triplicate. The plates were incubated at 37°C for three days.

Subsequently, the his^+ and trp^+ revertant colonies were counted. If the mean number of his^+ or trp^+ revertants was two fold or greater, the background lawn of bacterial growth was examined microscopically to determine any growth-diminishing or growth-enhancing effects by the test substance. Cytotoxicity was defined as a reduction in the number of revertant colonies and/or a clearing of a background lawn. The assay was considered to be negative and the test substance non-mutagenic if there were no dose-related increases in the mean number of revertant colonies, and a

reproducible positive response at any of the test points [Van Delft, 1999 Vol 5 Tab 76].

No statistical analysis was performed for this test. The apparent decrease in the mean number of revertant colonies for the *Escherichia coli* strain in the presence of the S9 mix at the highest dose level did not, according to the authors, indicate a toxic effect but was probably attributable to difficulties in discerning revertant colonies from the high background lawn of bacterial growth. In the 3rd assay the authors noted a slightly more dense background lawn of bacterial growth at most test substance dose levels, and a very dense background lawn of bacterial growth was observed for the *Escherichia coli* strain at the highest dose level. The observations were conducted microscopically.

Results showed that Hayashibara isoamylase did not cause a reproducible two-fold or greater increase in the mean number of revertant colonies appearing in the test plates, as compared to the background spontaneous reversion rate observed with the control, nor did it produce a dose-response in either the absence or the presence of the S9-mix [Van Delft, 1999 Vol 5 Tab 76]. The study report stated that the positive controls produced the expected increase in the number of his^+ or trp^+ revertant colonies in both the absence and the presence of the metabolic activator. The authors concluded, that Hayashibara isoamylase was not mutagenic under the conditions employed in the study, and it was not toxic to any strain of bacteria tested.

4. *In Vitro* Mammalian Chromosome Aberration Test In Cultured Human Lymphocytes

Hayashibara isoamylase was evaluated for its potential to induce chromosome aberrations in cultured human lymphocytes [Haddouk, 2001 Vol 2 Tab 20]. The isoamylase was tested in two independent experiments both with and without a metabolic activator (S9). The S9 mix was prepared from a rat microsomal liver fraction, which had been induced with Aroclor 1254.

Two studies were performed using concentrations of isoamylase ranging from 156.25 to 5,000 ug/ml. Doses for scoring chromosomal aberrations were selected on the basis of cytotoxicity as evidenced by a reduction in the mitotic index (MI) [Haddouk, 2001 Vol 2 Tab 20]. Each culture consisted of heparinized whole blood, which was added to a culture medium containing phytohemagglutinin (a mitogen). Cultures were incubated at 37°C under controlled humidity of 5% CO_2/95% air for 48 hours.

In the first experiment the lymphocyte cultures were exposed to either the isoamylase or the control substance with or without metabolic activation (S9) for a period of 3 hours. Cultures were rinsed, and the cells were harvested 20 hours after the initiation of the treatment (approximately 1.5 cell cycles). One and one half-hours before harvest the cultures were arrested at the metaphase stage of mitosis with a 10 μg/ml solution of colcemid. The results of this experiment showed no mutagenesis.

Since the first experiment revealed no mutagenic potential for the isoamylase, a second test was conducted with or without the S9 mix. In the absence of the metabolic activator, the cells were continuously exposed to the test or control materials. As in the first experiment, when the metabolic activator was used, the cells were exposed for 3 hours and then rinsed. The treated lymphocyte cultures were harvested at either 20 (about 1.5 cell cycles) or 44 hours after the initiation of treatment. As before, 1.5 hours before harvest the cultures were treated with a 10 μg/ml solution of colcemid to arrest cell maturation at metaphase. Cells from both experiments were fixed, stained with Giemsa and coded for scoring [Haddouk, 2001 Vol 2 Tab 20].

With a treatment volume of 100 μl/5.5 ml culture medium, the treatment concentrations both with and without S9 mix were as follows: 156.25, 312.5, 625, 1250, 2500, 3750, and 5000 μ/ml for the first experiment. Identical concentrations were used in the second experiment, except that the 3750 μ/ml dose was omitted [Haddouk,

2001 Vol 2 Tab 20].

Examination of the lymphocyte cultures treated with negative or positive controls had chromosomal aberrations within specified and acceptable limits. At the dose of 5,000 ug/ml no precipitate was observed in the medium, the pH and osmolality were equivalent to negative control cultures, and the treatment sample was non-toxic and freely soluble. These findings strongly suggested that the study was performed in an appropriate manner and that the results were valid.

Slight or sporadic decreases in the mitotic indexes were observed in some cultures; however, since no dose-related pattern was seen these finding were not considered significant. The authors concluded that under their experimental conditions, isoamylase from *Pseudomonas amyloderamosa* did not induce chromosome aberrations in cultured human lymphocytes [Haddouk, 2001 Vol 2 Tab 20].

5. Isoamylase in Human Serum

Isoamylases are normal products of several organs of the human body, including the pancreas, salivary glands, liver, and the genital tract. In 1975 Skude published a report describing the sources of serum isoamylase and their normal distribution by age [Skude, 1975 Vol 4 Tab 59]. Recently, the determination of pancreatic isoamylase levels has become important in the clinical determination of pancreatic and other diseases [Skude, 2000 Vol 4 Tab 64]. As a result various researchers have examined the secretion of isoamylases and have determined their origins by organ.

In a paper published in French, Carlier *et al.* identified α-amylases from parotid secretions in human saliva. The authors conducted comparison studies of amino acid sequences and indicated that separation of isoamylases would be of great interest in clinical biology in the diagnosis of diseases related to body organs, which produce isoamylase [Carlier, A. *et al.*, 1973 Vol 2 Tab 11].

Nishimura *et al.* studied the clinical implications of the distribution of salivary type (S-type) isoamylase activity, as compared with that of pancreatic type (P-type) isoamylase activity in the serum of 18-23 year old female adults [Nishimura, *et al.,* 1988 Vol 3 Tab 50]. The group found that a high correlation existed between S-type isoamylase levels determined on the same subjects after a one year interval. Further, the serum level of S-type isoamylase was highly correlated with the S-type isoamylase activity in saliva. The article reported that there was a weak positive correlation between energy intake and the total and S-type isoamylase activities in serum. However, they found no negative correlation between the S-type isoamylase level and body mass index (BMI), as had been reported for young male adults [Nishimura, *et al.,* 1988 Vol 3 Tab 50].

Levitt *et al.* found that normal serum contains three major isoamylases with isoelectric points of pH 6.9-7.1, 6.3-6.6, and 5.9-6.1 [Levitt, *et al.*, 1977 Vol 3 Tab 40]. The investigators reported that pancreatic isoamylase comprised 32% of serum amylase of normal subjects. The other two major serum isoamylases corresponded to the two major isoamylases of saliva. Salivary isoamylases were increased in patients with hyperamylasemia secondary to mumps or parotid tumors. The isoamylase present in serum in elevated concentration in acute pancreatitis, and the only isoamylase found in duodenal drainage or in pancreatic homogenates had an isoelectric point of 6.9-7.1. This isoamylase was virtually absent from the serum of patients with pancreatic exocrine insufficiency. The isoamylase found in liver, fallopian tube, and sweat generally had a lower isoelectric point than the pancreatic isoamylase and was not found in the serum [Levitt, *et al.*, 1977 Vol 3 Tab 40].

Ihse *et al.* reported that the concentration of trypsin, pancreatic isoamylase, phospholipase, and lipase were detected in intestinal content during the first two hours of digestion of a test meal. In normal subjects the concentration curves for all enzymes displayed a typical biphasic pattern [Ihse, *et al.,* 1977 Vol 3 Tab 29]. In patients with chronic pancreatic disease, the typical enzyme concentrations

were markedly diminished.

Skude *et al.* found that the pancreatic serum isoamylases could be separated into a main fraction and more anodally migrating minor fractions on agarose gel electrophoresis [Skude, *et al.,* 1977b Vol 4 Tab 62 and Skude, *et al.,* 1976a Vol 4 Tab 60]. In acute pancreatic hyperamylasemia as in normal subjects the activity of the anodally migrating pancreatic amylase did not exceed 20% of the activity of the main fraction. In patients with pancreatic main duct occlusion, or stenosis, the anodally migrating pancreatic amylase increased proportionally more than the main fraction, and could even be detected at a higher level than the main fraction. The majority of these patients also had abnormally increased total pancreatic serum amylase activity.

Attempts by Palani, *et al.* to differentiate hyperamylasemia caused by severe, acute ethanol pancreatitis from hyperamylasemia caused by other acute abdominal diseases were reported to be unsuccessful, when serum and urine pancreatic isoamylase determinations were compared with the amylase to creatine clearance ratio, because the data overlapped by 44 and more than 80%, respectively [Palani, *et al.*, 1977 Vol 4 Tab 54].

Skude *et al.* found that homogenates of tissue from the female genital tract contained isoamylases, which were, to a certain extent, electrophoretically distinguishable from pancreatic and salivary isoamylases. In healthy nonpregnant women, high levels of activity of genital isoamylases were found in tissue homogenates of cervical and fallopian tube mucosa, whereas the activity was weak or absent in homogenates of endometrium [Skude, *et al.,* 1976b Vol 4 Tab 61]. The isoamylases of the cervical mucosa had an electrophoretic migration rate toward the anode identical to that of the salivary main fraction, whereas the isoamylases of the fallopian tube migrated faster. Specific genital isoamylase activities were also demonstrable in peritoneal fluid collected from the cul-de-sac. During the menstrual cycle, these activities showed a midcycle peak. The group also reported that in pregnant women, the levels of activity of the genital

isoamylases in peritoneal fluid were lower than in nonpregnant women. In homogenates of the male accessory genital glands, the isoamylases specific for the genital tract were present in minute amounts. The isoamylases specific for the genital tract were not detectable in the serum of either sex [Skude, *et al.,* 1976b Vol 4 Tab 61].

Berk *et al.* measured serum total amylase activity, serum pancreas type isoamylase activity, and the ratio of pancreas-type isoamylase relative to creatinine in the urine of patients with clinically suspected chronic pancreatitis and pancreatic insufficiency [Berk, *et al.*, 1979a and b Vol 2 Tabs 9 and 10]. The authors concluded that subnormal values for pancreas-type isoamylase in the serum and urine are supportive but not definitive evidence for pancreatic insufficiency.

Pancreatitis diagnosis by serum pancreatic isoamylase determination and by serum trypsin determination using radioimmunoassay were compared in patients diagnosed as having pancreatitis by the secretin-pancreozymin test [Lankisch, *et al.,* 1980 Vol 3 Tab 38]. Diagnoses were correct for chronic pancreatitis in 61, 60, and 46% of the cases when based on the pancreatic isoamylase inhibitor test, pancreatic isoamylase determined by electrophoresis, and trypsin determination. In cases of acute pancreatitis, higher accuracies were obtained (70, 66.6, and 53% with the same 3 tests, respectively) [Lankisch, *et al.,* 1980 Vol 3 Tab 38].

Balldin *et al.* analyzed the secretory pancreatic proteins in serum in a group of cigarette smokers and a control group of nonsmokers before and after i.v. secretin stimulation. None of the persons tested had any signs of pancreatic disease. Concentrations of pancreatic proteins, (serum total amylase activity, pancreatic isoamylase, cationic trypsinogen, and pancreatic trypsin inhibitor), in all the cigarette smokers increased from normal to abnormally high serum concentrations after secretin stimulation, while normal subjects showed no change. The investigators suggested that this finding was a probable toxic effect of cigarette smoking on the exocrine pancreas [Balldin, *et al.,* 1980 Vol 2 Tab 5].

6. Acceptable for General Use in Foods

a. Expert Panel Opinion

Hayashibara International Inc. convened an Expert Panel qualified by scientific training and experience to review the data and information available on the safety and use of isoamylase. The Expert Panel met in Washington, D.C. on November 16, 2000 and conferred further by telephone. Subsequently, the Expert Panel signed an opinion, concurring with the Hayashibara International Inc., that its isoamylase could be considered generally recognized as safe. The opinion of the Expert Panel is attached at Appendix A.

b. Product Volume

Hayashibara Company, Ltd., Okayama, Japan has been manufacturing a commercial preparation of isoamylase since 1979. Cumulatively, Hayashibara has used approximately 300 tonnes of their isoamylase enzyme preparation to produce various foods for the Japanese and other Asian markets. Additionally, the enzyme preparation is currently used to manufacture a high-purity maltose drip for intravenous use [Hayashibara, 2000, Unpublished data Vol 2 Tab 25]. Table 11 below shows the sales history of the preparation from 1979 through 2000.

Table 11

Isoamylase Use		
≥1,250,000 U/g		
Year	Volume (kg)	Cumulative Total (kg)
1979	1,868	
1980	4,314	6,182
1981	5,566	11,748
1982	4,739	16,487
1983	7,848	24,335
1984	9,276	33,611
1985	8,741	42,352
1986	6,968	49,320
1987	10,342	59,662
1988	6,616	66,278
1989	10,243	76,521
1990	10,982	87,503
1991	10,841	98,344
1992	10,933	109,277
1993	11,270	120,547
1994	10,730	131,277
1995	14,460	145,737
1996	21,779	167,516
1997	32,653	200,169
1998	32,010	232,179
1999	43,181	275,360
2000	26,689	302,049
Reported by Hayashibara Okayama Plant II		

c. Employee Exposure

Employees of the Hayashibara Company, Ltd. who have been continually in direct contact with either *Pseudomonas amyloderamosa* or the isoamylase enzyme preparation have reported no allergic symptoms or other adverse reactions to the company clinic, since production began in 1979 [Fujiwara, 2000 Vol 2 Tab 19]. It is assumed that persons directly involved in the enzyme production would be subject to constant exposure, even if at minimal levels.

7. Analysis of Five Production Lots of the Commercial Preparation

In order to demonstrate their ability to manufacture an isoamylase enzyme preparation consistently over a period of time (March to August, 2000), Hayashibara Company, Ltd., analyzed 5 lots in duplicate against the variables shown in Table X below. The results showed that the average activity per gram was 1,351,200, and the average pH was 4.23. The average viscosity and specific gravity at 20°C were 6.56 and 1.18, respectively. Heavy metals as lead and arsenic as arsenic oxide were both below the level of detection. No cells from *P. amyloderamosa* were present.

Table 12
Analysis of Five Lots in Duplicate

	Lot Number					
Variable	90305	90402	90521	90702	90806	Mean of Means
Activity (U/g)	1,362,000	1,362,000	1,862,000	1,367,000	1,330,000	1,351,200
PH	4.17	4.08	4.2	4.40	4.2	4.23
Viscosity (cP, 20°C)	6.1	5.8	5	5.7	6	6.56
Specific Gravity (20°C)	1.175	1.174	1.169	1.195	1.180	1.18
Heavy Metals (as Pb)	≤ 30 ppm	≤ 30 ppm	≤ 30 ppm	≤ 30 ppm	≤ 30 ppm	≤ 30 ppm
Arsenic (As As_2O_3)	≤ 1 ppm	≤ 1 ppm	≤ 1 ppm	≤ 1 ppm	≤ 1 ppm	≤ 1 ppm
P. amyloderamosa	Negative	Negative	Negative	Negative	Negative	Negative
Date of Manufacture	March 5, 2000	April 2, 2000	May 21, 2000	July 2, 2000	August 6, 2000	

8. Identification of Impurities

a. Analysis of Heavy Metals

Hayashibara Company, Ltd. has analyzed its product for the presence of metal or other impurities in its enzyme product. The results for three lots analyzed in duplicate are shown below in Table 13. A second Table (Table 14) depicts the mean of means for the same three lots. The results showed that levels of cadmium, lead and arsenic were less than 0.1 ppm, and that levels of mercury were below the level of detection at <0.005 ppm.

Table 13
Analysis of Three Lots of Hayashibara Isoamylase
For Heavy Metals
(Average of Duplicate Analyses)

Element	Assay Method	Detection limit (mg/kg)	Lot 91022 10/22/1999	Lot 91119 11/26/1999	Lot 00218 02/18/2000
Cadmium	AAS[1]	0.001	0.0010	0.0010	<0.0010
Mercury	XPT-90 113-2	0.005	<0.005	<0.005	<0.005
Lead	AAS[1]	0.005	0.014	0.020	0.013
Arsenic	AAS[1]	0.005	0.013	0.007	0.016

[1]AAS: Atomic Absorption Spectrometry

Table 14
Hayashibara Isoamylase
Heavy Metals
(Mean of Means)

Metal	(mg/Kg)
Cadmium	0.001
Mercury	<0.005
Lead	0.016
Arsenic	0.012

b. Mycotoxin Levels

Since *Pseudomonas amyloderamosa* is used to produce sugars from starch, the Hayashibara Company, Ltd. measured the level of mycotoxins in their isoamylase preparation. All mycotoxins tested were below the level of detection, as shown in Table 15 below.

Table 15
Mycotoxin Levels of Hayashibara Isoamylase

Mycotoxin	Assay Method	Mycotoxin Concentration (μg/g)
Aflatoxin B1	ISO 14718	<0.001
Aflatoxin B2	ISO 14718	<0.001
Aflatoxin G1	ISO 14718	<0.001
Aflatoxin G2	ISO 14718	<0.001
Sterigmatocystine	HPLC	<0.005
Zearalenone	HPLC	<0.02
Ochratoxin	HPLC	<0.001

c. Impurities Specific to the Manufacturing Procedure

Microbiological analyses of the Hayashibara isoamylase commercial preparation are summarized in Table 16 below. The results showed that Total Mesophilic bacteria counts, thermoresistant spores, and flat sour spores were less than 100, per gram, and that levels of coliforms, *Salmonella, sp., Listeria,* and coagulase positive *Staphylococcus* were less than the level of detection.

Table 16
Microbial Analyses of Hayashibara Isoamylase

Microbial Variable	CFU/g
Total Mesophilic Bacteria	< 100
Coliforms	< 1
Salmonella sp./25 g	Negative
Listeria/25 g	Negative
Coagulase Positive	< 1
Thermoresistant spores	< 100
Flat sour spores	< 100

d. Specifications for Hayashibara Isoamylase

In accordance with the data presented above, Hayashibara Company, Ltd. has set specifications for its commercial product. Those specifications are shown below in Table 17.

Table 17
Commercial Specifications for Hayashibara Isoamylase

Appearance	Yellowish brown liquid
Activity	≥1,250,000 U/g
Arsenic	<0.1 ppm
Lead	<0.1 ppm
Cadmium	<0.1 ppm
Standard Plate Count	< 100 CFU/g
Salmonella sp.	Negative/25g
Coliforms	< 1

e. Data to demonstrate that the enzyme preparation is free of biologically significant levels of toxins, antibiotics, and transformable DNA which could code for toxins or proteins known to inactivate therapeutic antibiotics.

Hayashibara isoamylase is produced by separating and purifying the enzymatic secretions of *Pseudomonas amyloderamosa*, a naturally occurring, non-pathogenic, non-toxigenic organism. No genetic manipulation of the organism's DNA has been performed, and no DNA from other sources has been added. Further, no antibiotics are used in either the growth or the purification of the isoamylase (See Section II above).

Section IV. Intended Use and Use Levels

A. Identification of All Foods in Which an Enzyme Preparation Is Intended to be Used.

1. Use as a Debranching Enzyme

Isoamylase is used as a debranching enzyme to produce such foods as starch sugars, including: glucose, maltose, trehalose, maltotetraose, dextrins, and oligosaccharides [Yokobayashi, *et al.*,1970 Vol 5 Tab 79, Yokobayashi, *et al.*,1973 Vol 5 Tab 80 and Sun *et al.*, 1999 Vol 4 Tab 66]. It may also be used to modify the properties of starches, including modified starch and amylopectin [*Federal Register* Vol 66, No. 63, April 2, 2001 Vol 2 Tab 17]. Isoamylase has recently been approved as a food additive at 21 CFR §172.892 for use in the modification of food starches.

2. Use in Foods

Research involving the use of isoamylase to achieve certain properties in foods has been ongoing for approximately three decades. Food scientists have been actively filing patents in which isoamylase is listed as a preferred substance. A Table showing the various and potential uses of isoamylase is shown below. These uses range from the modification of starch to produce low-fat ice cream to the modification of bean paste for the production of a bean flavoring [US Patent 5,962,047 Vol 4 Tab 72 and US Patent 5,773,055 Vol 4 Tab 67].

Table 18

Uses of Isoamylase as a Debranching Enzyme in Foods

PRODUCT	TOS/g	PATENT NUMBER	DESCRIPTION
Enzyme–Resistant Starch for Reduced-Calorie Flour Replacer	~0.0016 mg/g	6,013,299	An enzyme-resistant starch with a high temperature of gelatinization, for reduced calorie flour suitable for baking. The preparation exhibits superior baking characteristics: cookie spread, golden brown color, pleasant aroma, and surface cooking.
Starchy Products	~0.041 mg/g	6,090,594	A starch composition with a high proportion of resistant starch structures to be used as a low calorie bulking component in food.
Hypocariogenic Hydrogenated Starch Hydrolysate	~0.081 mg/g	5,493,014	A sweetener, consisting of a hypocariogenic hydrogenated starch hydrolysate for use in sweets, chewing gums, toothpastes, and drinks.

Table 18 (Continued)

Microcrystalline Starch Based Product	~.0687 mg/g	5,962,047	A microcrystalline starch based product with a particle size less than 10 microns. Used in reduced-calorie foods, such as reduced-fat and no-fat ice cream.
Non-Reducing Oligosaccharides	~0.0015 mg/g	5,780,620	A non-reducing oligosaccharide with reduced sweetness, appropriate viscosity, and the ability to retain moisture. Used in foods, molded foods, and beverages.
A Bean Flavor	~41.0 mg/g	5,773,055	A source of reducing sugars from the bean, to provide a high-strength oil-soluble fried bean flavor.
A Starch Emulsifier Composition	~0.81 mg/g	6,017,388	A starch-emulsifier composition to be used to prepare lactose-free dairy products, reduced-fat, and fat-free foods, especially cheeses, and as a texturizing agent in dairy products. May also be used as an opacifier in milk to improve the visual appearance of food.
Manufacture of Trehalose	~0.0454 mg/g	Subject of GRN 000045	A multifunctional carbohydrate. The enzyme is inactivated during the process.

Based on the examples listed above in Table 18 and the TOS value of the enzyme preparation, it was possible to calculate a table showing the average intake of isoamylase TOS by food group. The results are shown below in Table 19. As an example, the average intake of Hayashibara isoamylase in baked goods is anticipated to equal approximately 0.023 mg/g of food. The most concentrated use of isoamylase cited is in the modification of

legumes (41.0); however, in this case isoamylase is used to modify a legume paste to create a flavor [US Patent 5,773,055 Vol 4 Tab 67]. Therefore, the intake of the enzyme TOS from such a process would be minimal. The highest intake of isoamylase may be found in milk products although essentially all of the enzyme would be inactivated and removed in the production process (See Table 19 below).

Isoamylase is also used as a process aid and is inactivated and removed in conventional starch hydrolysis processes, which result in the production of starch-derived sugars. As an example, approximately 0.0454 mg of isoamylase preparation (≥ 1,250,000 U/g) is used in the production of 1.0 gram of trehalose. Essentially all of the enzyme is inactivated and removed in the production process [GRAS Notice No. 45, 2000 Vol 5 Tab 83].

Table 19
Average Total Organic Solids Contributed by Hayashibara Isoamylase in Various Foods

Food Categories	Average TOS/g of Food (mg/g)
Baked Goods	0.023
Legumes	41.0
Milk products	0.75
Sweets	0.043
Chewing Gum	0.081
Non-Alcoholic Beverages	0.043

3. Fate of the enzyme preparation in food

Typically, enzyme preparations are inactivated during food cooking and processing, since they are denatured at temperatures exceeding 160°F (~72°C) [Lehninger, 1982, Vol 3 Tab 38]. In order to demonstrate complete denaturation of the enzymes used in the production of Hayashibara trehalose, the

company performed an ELISA assay to test for residual enzymes in the final trehalose product [Hayashibara, 2000, Unpublished data].

To establish the ELISA system, rabbits were immunized with all seven (7) of the enzymes used in trehalose production. The investigators, then confirmed subsequent increases in serum antibody titer to the seven immunogens tested. A 50% solution of trehalose (Lot No 511031) was analyzed by ELISA to demonstrate the absence of any residual enzymes in the final product. Results showed that the heat to which trehalose had been exposed during processing and drying (>70°C) was sufficient to denature all seven of the enzymes used in production. Therefore, to generalize from the example given above, under conditions in which a food product is subjected to heat above the denaturation temperature for the enzyme, it is unlikely that enzyme activity can be detected in the final food product.

Table 20
Residual Enzyme Content in Hayashibara Trehalose

Immunogen	Lot No	ELISA Detectable Range	Analytical Results
Thermamyl 120L	AXNO4086	1.0-100 ng/ml	< 1.0
Spitase HS	261467	1.0-50 ng/ml	< 1.0
Neospitase PK-2	2614841	0.5-50 ng/ml	< 0.5
HT Enzyme	70204, 70212	50-1,000 ng/ml	< 50
TC Enzyme	61211	2.5-250 ng/ml	< 2.5
Isoamylase	60911, 61011, 61018	50-1,000 ng/ml	< 50
Glucoamylase XL-4	2607753	1.0-100 ng/ml	< 1.0

Section V. Residues of Immobilized Enzymes

This section is not applicable, since Hayashibara isoamylase is not immobilized.

1. Determination of the amount of enzyme preparation which migrates into food from an immobilized enzyme preparation (if applicable)

2. Quantitative evaluation of other components of an immobilized enzyme preparation migrating into food
   a. Carriers
   b. Immobilizing agents
   c. Flocculants
   d. Immobilizing agents
   e. Impurities

3. Quantitative analysis of immobilized enzyme components into food or into solvents that simulate the food

Section VI. Technical Effects

1. Description of methods for using the enzyme preparation in food

In 1930, Nichimura reported that yeast extracts contained an enzyme, tentatively named amylosynthase, which showed starch liquefying activity, and accelerated the action of other amylases. This enzyme was reported to differ in heat sensitivity from the α- and β-amylases. Further studies by Nishimura and Minagawa showed that on glutinous rice starch, the enzyme caused an increase in the iodine color reaction. The enzyme was therefore regarded as a starch-synthesizing enzyme [Okada, 1987 Vol 4 Tab 53].

Later the enzyme action on rice starch was re-examined by Maruo and Kobayashi, who found that the reaction products were smaller in molecular weight than the original starch, and stained bluish-purple with iodine. Also, the reaction mixture showed a tendency to produce precipitates of retrograded starch. These phenomena were considered to be due to an extensive debranching of starch and thus, the name "isoamylase" was proposed for the enzyme [Okada, 1987 Vol 4 Tab 53].

The most important application of the enzyme is for the industrial production of glucose or maltose in combination with glucoamylase or β-amylase. Using isoamylase allows for the production of high purity maltose. Because of this high purity, an efficient process was developed to produce maltitol, which is an important polyol in the food industry. Recently, 6-O-α-maltosyl-, and 6-O-α-maltotriosyl-cyclodextrins were reported to be produced in high yields by applying the condensation reaction or transfer reaction of an enzyme system using isoamylase [Okada, 1987 Vol 4 Tab 53].

Table 18 shows that isoamylase has been used by food scientists for modifying the properties of starchy food ingredients to prepare low-fat or no-fat formulations, with textural characteristics similar to their full-fat counterparts. Other patents have been filed on the use of isoamylase to produce various types of sweeteners from starch [US

Patent 5,493,014 Vol 4 Tab 71]. Hayashibara Company, Ltd. also produces a high-purity maltose, which has been used in Japan as an intravenous drip [Hayashibara Company Literature, 2000 Vol 3 Tab 26].

2. Evidence to demonstrate that the enzyme performs its intended function in food

Recently, food scientists have actively considered and patented the use of isoamylase in various food preparations. A discussion of the uses of isoamylase to improve the qualities of starch-containing products is presented below.

US Patent 5,493,014

US Patent 5,493,014 describes the process for preparing hypocariogenic hydrogenated starch hydrolyzates, and provides examples of their application [US Patent 5,493,014 Vol 4 Tab 71]. This hydrolysate is characterized as unique, in that it is comprised of 0.1 to 19% by weight of sorbitol, 35 to 87% by weight of maltitol, and 1 to 17% by weight of polysaccharides, which are not hydrolyzed by any amyloglucosidase. The remainder of the product consists of hydrogenated oligo- or polysaccharides. According to the inventor, the hydrolysate can be used as a sweetening agent in products intended to be ingested by humans or animals. It is especially useful in sweet chewing gums as well as in syrups, food, or pharmaceutical elixirs [US Patent 5,493,014 Vol 4 Tab 71].

In this technology isoamylase is used to hydrolyze the α-1-6 bonds of amylopectin. The action of isoamylase allows only the polysaccharides that are very difficult to digest enzymatically to remain in the hydrolysate. The application of the enzyme in the food formulation is approximately 0.081 mg/g [US Patent 5,493,014 Vol 4 Tab 71].

US Patent 5,962,047

US Patent 5,962,047 teaches the process for producing a microcrystalline starch-based product and its use in foods. The product consists of microcrystalline starch, glucose and short chain glucooligosaccharides having an average particle size of less than about 10 microns [US Patent 5,962,047 Vol 4 Tab 72]. The process comprises debranching, retrograding, and hydrolyzing a starch. The debranching enzyme was added at the rate of approximately 0.687 mg/g of starch. One food formulation that could be prepared using the ingredient is no-fat ice cream. The formulation and procedure for preparing the ice cream are shown below.

Preparation of No-Fat Ice Cream

Ingredient	Amount (Kg)
Skim milk	23.24
Non-fat dry milk	2.04
Sugar	3.86
Maltodextrin (5 DE)	1.45
Corn syrup solids (36 DE)	0.83
Stabilizer	0.08
Mono- & diglycerides	0.06
Microcrystalline starch	0.26
Based product total	31.82

No-fat Ice Cream Procedure

The dry ingredients were dispersed in the liquid using an overhead stirrer for approximately five minutes. The mixture was pasteurized in a regenerating plate heat exchanger (APV/Crepaco) in which the pre-mix was heated to 71 C° in the regeneration portion of the heat exchanger and homogenized through a 2-stage homogenizer [first stage 2000 psi / second

stage 5000 psi (APV/Gaulin, Wilmington, MA)] followed by pasteurization at 85 C° for 25 seconds. The mixture was then cooled to 4°C [US Patent 5,962,047 Vol 4 Tab 72].

The ice cream pre-mixes were frozen in a continuous ice cream freezer (PMS, Philadelphia, PA) operating at -7°C, packed in 946 ml containers and hardened overnight at -40°C [US Patent 5,962,047 Vol 4 Tab 72].

US Patent 5,773,055

US Patent 5,773,055 teaches the process for preparing a fried bean flavor. The process involves preparing a bean paste, hydrolyzing the bean paste with a proteinase and a carbohydrase, and reacting the hydrolyzed bean paste at an elevated temperature with an edible fat or oil and lecithin [US Patent 5,773,055 Vol 4 Tab 67].

In this method isoamylase is used as a carbohydrase to provide a source of reducing sugars from the bean. The enzyme is added to the substrate at approximately 41 mg/g [US Patent 5,773,055 Vol 4 Tab 67].

US Patent 6,056,980

US Patent 6,056,980 teaches that a starch sugar composition can be produced using an α-amylase, an oligosaccharide-forming amlyase, β-amylase, a debranching enzyme and an enzyme capable of forming nigerooligosaccharide by transglycosylation or a condensation reaction. The substrate and enzymes are allowed to react successively or simultaneously. The substrates may be starch, a maltooligosaccharide with a degree of polymerization not lower than maltose, or a dextrin. Nigerooligosaccharides are allowed to form, which may be further reduced to obtain a starch sugar composition containing the nigerooligosaccharide. This starch sugar composition may be added to food and beverages as portion of the sweetener system. The inventors claim various

advantages can be provided by the product, including am improvement in the quality of the sweetness [US Patent 6,056,980 Vol 4 Tab 68].

Isoamylase is used as a debranching enzyme to produce a starch sugar composition which:

- imparts sweetness
- improves the quality of sweetness
- improves the moisture retention of foods

A process example is provided by the inventors as follows:

1. liquefy corn starch using α-amylase to produce starch liquefying solution (concentration 30% by weight) with a glucose equivalent of 7
2. adjust pH to pH 5

3. based on 1 part by weight of starch add:
   - 0.001 part by weight of β-amylase
   - 0.0001 part by weight of isoamylase
   - react at 55 C° for 24 hours to obtain substrate.

4. Add 0.08 unit nigerooligosaccharide glucanotransferase (produced by an *Acremonium* strain) per gram of substrate. React at 55 ° C for 48 hours.

   Nigerooligosaccharide-containing syrup
   Product Composition

	%
Glucose	25.6
Maltose	22.9
Nigerose	(5.6)
Trisaccharide	23.1
(Nigerooligosaccharide)	(16.2)
Tetrasaccharide or higher	22.8
(Nigerooligosaccharide)	(11.2)
Total of nigerooligosaccharides	33.0

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Process for making enzyme-resistant starch for reduced-calorie flour replacer

US Patent 6,013,299

US Patent 6,013,299 describes a process for making enzyme-resistant starch for a reduced-calorie flour replacer [US Patent 6,013,299 Vol 4 Tab 69]. In this process an enzyme resistant starch, which has a melting point or endothermic peak of at least about 140°C, is produced in yields of about 25% by weight, based upon the weight of the original starch ingredient. A gelatinization stage, nucleation/propagation stage, and a heat-treatment stage are used to produce reduced calorie starch-based compositions, which contain the enzyme resistant starch. The enzyme resistant starch is produced using crystal nucleation and propagation temperatures, which avoid substantial production of lower melting amylopectin crystals, lower melting amylose crystals, and lower melting amylose-lipid complexes. The propagating temperature used is above the melting point of any amylose-lipid complexes but below the melting point of the enzyme resistant starch. The inventors state that the starch-based compositions comprising the high-melting resistant starch described in the invention exhibit unexpectedly superior baking characteristics, such as cookie spread, golden brown color, pleasant aroma, and surface cracking, which are all comparable to those achieved with conventional wheat flour. The high melting point of the enzyme resistant starch, as measured by DSC, permits its use in baked good formulations without substantial loss of enzyme resistance upon baking. In this invention isoamylase is used as a debranching enzyme to produce a precipitate from amylopectin and to increase the yield of enzyme-resistant starch [US Patent 6,013,299 Vol 4 Tab 69].

US Patent 6,090,594

US Patent 6,090,594 describes an invention, which relates to a

starch composition containing a relatively high proportion of so called "resistant starch" structures. The composition of the invention is obtained by debranching an aqueous slurry of a gelatinized starch and retrograding the resulting product [US Patent 6,090,594 Vol 4 Tab 70]. Isoamylase is used as a debranching enzyme to produce a starch composition with a high proportion of "resistant starch" structures. The product can then be used as a low calorie-bulking component in food. An example of the procedure is given below:

Preparation of "resistant starch" structures

a. Dissolve 25 kg potato starch maltodextrin (dextrose equivalent of 3) in water to achieve a 25% concentration by weight
b. Heat the suspension to 50 C°
c. Adjust to pH 5.0
d. Add 25 ml Isoamylase
e. As reaction proceeds for 20 hours, heat to 80°C
f. Adjust to pH 3.0 for 1 hour to deactivate isoamylase

The inventors reported that after debranching the average molecular weight of the oligomers was 13,680. The process was then continued as follows:

g. Readjust to pH 5.0
h. Cool to 4° C for 24 hours
i. Stir/dilute with water/spray dry to achieve a 15% suspension by weight

The product contained 28.9% resistant starch compared to 7.3% in a maltodextrin ** debranching [US Patent 6,090,594 Vol 4 Tab 70].

<u>US Patent 5,780620</u>

US Patent 5,780620 describes the production of a novel non-reducing oligosaccharide with a structure represented by the

formula α-D-oligoglucosyl α-D-oligoglucoside. The oligosaccharide may be reacting with either an aqueous solution which contains trehalose and an α-glucosyl saccharide or an aqueous solution which contains a non-reducing saccharide with a trehalose structure at its end to a saccharide-transferring enzyme. The oligosaccharide thus produced has a reduced sweetness, superior stability, appropriate viscosity and less susceptibility to crystallization. The oligosaccharide was also described as having superior solubility when in crystalline form. These properties according to the inventors make the oligosaccharide very useful in various compositions including foods, beverages, cosmetics, and pharmaceuticals [US Patent 5,780620 Vol X Tab X].

In this technology isoamylase is used as a debranching enzyme to accumulate maltosyl maltoside, maltotriosyl maltoside, and maltotriosyl maltotrioside. The product is used at the rate of 50 units per gram of starch in a 20% starch suspension [US Patent 5,780,620 Vol 4 Tab 73].

US Patent 6,017,388

US Patent 6,017,388 teaches a method of producing unique starch-emulsifier compositions by heating a starch in the presence of an emulsifier to form a complex [US Patent 6,017,388 Vol 4 Tab 74]. The product can be further modified to yield a composition containing more than 20% short chain amylose. The starch-emulsifier composition can be manufactured in the form of powders, gels, or pastes. The amount of the composition used in food or beverages varies from 1-10% by weight. The product can be used

- in the preparation of reduced-fat and fat-free products
- in preparation of lactose-free dairy products
- as a texturizing agent in various dairy products
- as an opacifier in milk to improve the visual appeal of food
- in fat-free, reduced fat and fat containing cheeses

The inventors described a general procedure, in which a starch emulsifier dispersion is treated with pullulanase or isoamylase at pH 3 to pH 7.5 for 1 to 30 hours at 55° C to 65° C.

The inventors explained that they were able to produce an opaque paste with a short and smooth rheology, which resembled Crisco® brand shortening, after enzymatically debranching a 15% corn starch slurry containing glycerol monostearate (3% on the weight of starch). This was in contrast to a more or less elastic and less opaque gel (like Jello®), which formed when the slurry had not been debranched [US Patent 6,017,388 Vol 4 Tab 74]. Material, which had not been debranched, had no low molecular weight fraction, and the particle size was 10.5μ at the 90th percentile. The usage level of the debranching enzyme was approximately 0.81 mg/g. The inventors provided a number of formulations for reduced-fat products using the starch emulsifier composition [US Patent 6,017,388 Vol 4 Tab 74].

Reduced-Fat Cake

A 50% reduced-fat cake was prepared with the starch-emulsifier product using the following formulation:

	Weight Percentage	
Ingredients	Control	Starch-Emulsifier Composition
Cake flour	27.63	27.63
Sugar	27.63	27.63
Baking powder	1.38	1.38
Salt	0.55	0.55
Skim milk	19.61	19.61
Shortening	11.05	5.525
25% Starch-emulsifier paste	0.00	5.525
Eggs	12.15	12.15

Procedure:

1. Prepare a 25% starch-emulsifier paste ahead of time by mixing 25 grams of the dried starch-emulsifier composition with 75 grams water and refrigerate overnight.

2. Cream sugar together with shortening and starch-emulsifier paste in a Kitchen Aid mixer.

3. Add sifted flour, salt and baking powder.

4. Mix for 1 minute on speed 1.

5. Scrape down sides of bowl and add eggs and milk.

6. Mix for 1 minute on speed 2.

7. Scrape down bowl and mix for 2 minutes on speed 3.

8. Bake at 350°F (177°C) for 25-30 minutes.

The characteristics of the reduced-fat cake were compared with the characteristics of the control in the Table below.

TABLE 21

	Control	Starch-emulsifier composition
Before baking:		
batter viscosity (1 = low, 5 = high)	4.0	3.5
After baking:		
height (cm)	4.2	4.3
weight (g)	146.93	176.22
volume (10 X 10 cm piece (cm^3))	420.00	430.00
density (g/cm^3)	0.3498	0.4098
weight (g) cake	533.85	650.00
Sensory evaluation		
	moist	slightly less moist
	tender	slightly less tender
	uniform crumb	uniform crumb
	good color	less color development
	good volume	good volume

50% Reduced-Fat Cream Cheese Spread

A 50% reduced-fat cream cheese spread was prepared with the starch-emulsifier composition (3% monoglyceride) prepared according to the following formulation:

50% Reduced-Fat Cream Cheese Spread

Ingredients	Weight Percentage
Full-fat cream cheese	48.09
25% Starch Emulsifier paste in skim milk	48.57
Pregelatinized starch	2.07
Salt	1.04
Cream cheese flavor	0.24

Procedure:

1. Blend 25% starch-emulsifier paste, pregelatinized starch, salt, and cream cheese flavor in a Kitchen Aid mixer until smooth.

2. Add full-fat cream cheese and blend until homogenous.

3. Fill into receiving container and refrigerate.

Fat-Free Onion Dip

A fat-free onion dip was prepared with the starch-emulsifier composition (3% monoglyceride) according to the following formulation:

Ingredients	Weight Percentage
15% Starch-emulsifier gel in skim milk	91.84
Dried onions	5.29
Hydrolyzed corn protein	0.91
Salt	0.64
15 X Starter distillate	0.50
Xanthan gum	0.26
Sour cream flavor	0.24
Citric acid	0.32

Procedure:

1. Blend 15% starch-emulsifier gel, starter distillate and xanthan gum with low shear until smooth.

2. Add corn protein, sour cream flavor, salt and citric acid and blend until homogenous.

3. Blend in dried onions until evenly distributed.

4. Pack and refrigerate.

Skim Milk

A skim milk was prepared with the starch-emulsifier composition (containing 3% sucrose stearate) according to the following formulation:

Ingredients	Weight Percentage
Skim milk	97.990
Starch-emulsifier powder	2.000
Carrageenan (FMC, Viscarin GP 109)	0.010

Procedure:

1. Slowly add the starch-emulsifier powder to 161°F (71°C) milk while mixing with a kitchen blender (high speed). Continue mixing for three minutes once dispersed.

2. Add carrageenan and mix for 1 minute.

3. Homogenize through a two-stage homogenizer (2500/500 psi).

4. Cool in ice/water bath in capped bottles to 50°F (10°C), shake intermittently.

5. Store refrigerated.

The milk prepared as described above was compared to skim milk and whole milk. The sensory evaluation was conducted by a three-person trained expert panel, and the results are given in the Table below.

Preparation of No-Fat Ice Cream

A no-fat ice cream was prepared with a starch-emulsifier-gum composition (SEC) using the following formulation:

Ingredients	Weight Percentage
Skim Milk	72.52
Corn Syrup Solids - 36DE	12.15
Sugar	2.60
Nonfat Dry Milk	6.47
Maltodextrin - 10DE	4.55
Powder (SEC)	1.20
Stabilizer Polmo (Germantown)	0.31
Mono-diglycerides, Gold Star Swan (Grinstad)	0.20

Procedure:

1. Funnel feed the dry ingredients, as a blend, into a recirculating stream of skim milk.

2. Preheat to 150°F (65°C) and homogenize through a 2 stage homogenizer; 2000 PSI (lst stage), 500 PSI (2nd stage).

3. Pasteurize at 185°F (85°C) for 25 seconds.

4. Age overnight at 40°F (4°C).

5. Add pure vanilla extract (2x) at 0.6% use rate, then freeze to 20°F (-6°C) with a continuous freezer (target 75% overrun).

6. Harden at -30°F (-32°C) for 24 hours (for quart size containers).

Preparation of No-Fat Mayonnaise

A no-fat mayonnaise was prepared with the debranched starch-emulsifier composition (SEC) (containing 2% monoglyceride) according the following formulation:

Ingredients	Weight Percentage
Water	65.39
Vinegar (50 grain, white distilled)	10.80
Sugar, granulated cane	4.50
Corn syrup solids (Frodex 24)	4.50
Powder (SEC) Corn starch Pregelatinized cornstarch	4.20
Egg yolks (frozen)	3.00
Soybean oil	2.50
Salt	2.30
Xanthan gum (Keltrol F)	2.00
Lemon juice concentrate	0.40
Potassium sorbate Sodium benzoate	0.20
β-carotene	0.10
TOTAL	0.01
	100.00

Procedure:

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1. Place water (185°F; 85°C) in glass vessel of a kitchen

blender.

2. Disperse debranched starch-emulsifier composition and corn starch as a dry blend. Slowly add the powders to the kitchen blender while mixing on maximum speed. Once dispersed, blend on high for 5 minutes to fully hydrate.

3. Add dry blend of xanthan, corn syrup solids, sugar and salt to kitchen blender, blend on high for 2 minutes, to fully disperse.

4. Transfer contents to a mini food processor, add egg yolk, color, acids and lemon juice, then process just enough to mix all ingredients.

5. Add oil, slowly, while mixing. Continue mixing for 1-2 minutes until product is smooth and homogeneous in appearance.

6. Fill containers, refrigerate overnight before evaluation.

Preparation of Reduced-Fat Peanut Butter Spread

A reduced-fat peanut butter spread was prepared with the debranched starch-emulsifier composition (SEC)(containing 2% monoglyceride).

Ingredient	Weight Percentage
Water	49.01
Peanut Butter (Skippy Full-Fat)	25.00
Maltodextrin (GPC M040)	15.00
Sucrose	2.50
Powder (SEC)	4.50
Glycerin	2.00
CMC (Aqualon 7MF)	0.50
Salt	0.50
Roasted Peanut Flavor	0.29

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(Bell#109-14081)	
Potassium sorbate (granular)	0.10
Caramel Color	
(Williamson #622)	0.60
TOTAL	100.00

Procedure:

1. Place ambient temperature water in glass vessel of a standard kitchen blender.

2. Slowly add starch-emulsifier composition and CMC (as a dry blend) while mixing on maximum speed. Mix for 5 minutes to fully hydrate.

3. Add sucrose, M040, salt, potassium sorbate (as a dry blend) and continue shear for 5 minutes.

4. Add premelted peanut butter, glycerin and color and continue mixing at medium speed for 1 minute. Transfer to a metal container; place in a boiling water bath (185-190°F; 85°C-88°C), and homogenize using a Silverson homogenizer at 3/4 maximum speed for 5 minutes (small emulsor screen). Add flavor last.

5. Place in ice water bath and cool to 80°F (27°C) while slowly mixing with an overhead stirrer. Measure Brookfield viscosity at 80°F (27°C).

6. Deaerate using a WhipMix deaerator.

7. Store refrigerated 2 to 3 days before evaluating.

Fat Free Chocolate Spread

A fat free chocolate spread was prepared with the debranched starch-emulsifier composition (SEC) (containing 2%

monoglyceride).

Ingredient	Weight Percentage
Maltodextrin M040	18.00
Water	50.56
Heavy Cream	7.90
Cocoa Powder (Bensdorp defatted)	3.50
Powder (SEC)	4.50
Xanthan Gum (Keltrol F)	0.04
Salt	0.50
Fructose (Krystar 300)	15.00
TOTAL	100.00

Procedure:

1. Prepare blend of dry ingredients.

2. Slowly disperse blend into 195-200.°F (90-94° C) water with a standard kitchen blender.

3. Blend on high speed for 5 minutes.

4. Add cream. Continue blending for 1 minute.

5. Deaerate using WhipMix deaerator.

6. Store refrigerated 2 to 3 days before evaluating.

Water and Oil Absorption

The water absorption (percent by weight) of the SEC was determined by a modification of American Association of Cereal Chemists Method 88-04. Instead of using 5 grams of

test sample and centrifuging at 2000 g, the inventors used 3 grams of sample dispersed in water and centrifuged at 1450 g. For oil absorption, commercial Wesson vegetable oil was used in lieu of water. The Table below shows the water and oil absorption results for the starch-emulsifier composition. Data for a debranched starch and a microcrystalline cellulose (Avicel PH105, FMC) are also presented for comparison [US Patent 6,017,388 Vol 4 Tab 74].

TABLE 22

Sample	Water Absorption (%)	Oil Absorption (%)
Starch-emulsifier composition	132	71
Debranched starch	211	86
Microcrystalline cellulose	213	137

3. Estimation or determination of the minimum level of the enzyme preparation required to accomplish the intended technical effect in food

   While exhaustive studies on the use of isoamylase to modify food ingredients has not been done, Harada, *et al* reported in 1972 that 0.01 mg of *Pseudomonas* isoamylase hydrolyzed the branching linkages in 1 gram of waxy maize amylopectin in 20 hours [Harada, *et al.*, 1972 Vol 2 Tab 22].

4. For immobilized enzyme preparations

   Not Applicable

   a. Measurement of the usable lifetime

b. Conditions of use and measurement of the amount of food processed per kilogram of the enzyme preparation
c. Discussion of any undesirable effects of the enzyme preparation on food
d. Discussion of factors that effect the functional properties of the enzyme in food

Section VII. Analytical Methodologies

A. Description of methods used to identify and evaluate the purity of the enzyme preparation

1. Hayashibara Company, Ltd. has developed a method for evaluating the purity of their isoamylase enzyme preparation. The equipment and reagents needed, as well as the analytical procedure are outlined below. Additionally, for clarity the calculation is repeated at number 4 below (originally presented on page 26).

a. Equipment

Heater:	100V (in Japan), 800-1000W
Thermal water bath:	Capable of retaining 40±0.1°C, with agitator, one unit Capable of retaining 15±1°C, with agitator, one unit
Spectrophotometer:	Designed for measuring coloration and turbidity optical path length: 10 mm

b. Reagents

Substrate solution

Accurately weigh 4.17 g of Lintner soluble waxy cornstarch, and suspend it with a small quantity (less than 50 ml) of pure water. Any starch remaining in the container should be washed out and added to the solution with a small quantity of pure water. Heat the suspension with an electric (or gas) heater and allow it to boil for 5 minutes. After immediate cooling with running water, add 100 ml of 0.5M acetic acid buffer solution (pH 3.5) and 100 ml of pure water to equal a total of 500 ml.

Enzyme solution

Dilute the enzyme with 0.01M acetic acid buffer solution (pH 3.5) to equal 25-50 U/ml.

0.01N Iodine solution

Dilute a 0.1N iodine solution 10 fold, (Iodine should have been stored in a brown glass bottle).

0.02N Sulfuric acid solution

2. Operation

Place 3.0 ml of the substrate solution in a test tube (18 Φ x 180 mm), and place the tube in a thermal water bath at 40±0.1°C for 10 minutes. Then, add 0.5 ml of the enzyme solution to this substrate solution and mix. 30 seconds after the addition of the enzyme solution add 0.5 ml of the mixture to 15 ml of 0.02N sulfuric acid solution, which has previously been added to a test tube (24 Φ x 200 mm), and mix. This solution should be designated as a blank solution.

The remaining enzyme-added reactant solution should be kept at 40.0±0.1°C. 30 minutes and 30 seconds after adding the enzyme solution, mix 0.5 ml of the reactant with another 15 ml of 0.02N sulfuric acid solution with agitation. This solution should be designated as a test solution. After both the blank and the test solutions have been allowed to stand at 25±1°C for more than 15 minutes, add 0.5 ml of 0.01N iodine solution to each of the solutions and mix well. Then allow the solutions to stand again at 25±1°C for 15 minutes. Measure absorbency at 610 nm in a 10 mm cell using pure water as a control. Designate the absorbency of the blank solution as E_{30}.

3. Definition of enzyme activity

Quantity of the enzyme, which is capable of increasing the absorbency by 0.008, is designated as one unit of activity.

4. Calculation

The enzyme activity is calculated in accordance with the following expression (Shown also on page 26).

Activity (u/g or u/ml) = $(E_{30} - E_o) / 0.008 \times 1/0.5 \times f \times$ (dilution ratio)

$= (E_{30} - E_o) \times 250 \times f \times$ (dilution ratio)

f: a factor of the substrate. A coefficient used to correct discrepancies among production lots of the substrate. The value is renewed each time the substrate is changed.

E_o: Absorbency of the blank solution (absorbency obtained at 30 seconds after initiation of reaction)

E_{30}: Absorbency of the test solution (absorbency obtained at 30 minutes and 30 seconds after initiation of reaction)

Activity of the commercial isoamylase product is about 1.25×10^6 U/g of enzyme solution.

B. Description of methods used to analyze food for the presence of the enzyme

No specific methods have been developed to analyze for the presence of the enzyme in food. In most foods undergoing heating, the enzyme would be degraded.

Section VIII. Exposure

A. Estimation on a chronic daily basis of the probable human exposure

Using commercial formulations prepared with isoamylase in other countries, examples from the recent relevant patent literature, and its own production of starch sugars Hayashibara has calculated the potential human exposure to Hayashibara isoamylase. The calculations were made by determining the total organic solids content of isoamylase utilized in each major starch-containing food category on a mg/g basis; averaging the total organic solids of isoamylase across all examples in each major food categories (See Table 1 below); and multiplying the average TOS value for the category by the estimated mean daily intake of that food category [Enns, *et al.*, 1999 Vol 2 Tab 14]. A sample calculation is given below:

Average TOS for formulations within each category X Mean Intake per day = Isoamylase TOS exposure per category

The exposure from each category was then added to yield a total potential mean exposure value. These calculations assume that isoamylase will be utilized in every food within a category, that all consumers are eaters, and that each food within a category is consumed on a daily basis. Therefore, the estimate shown in Tables 23 and 24 are extremely conservative. Further, multiplying the average mean exposure of 80.0 mg (given as the total possible intake in Table 25) by 2 to estimate the mean exposure at the 90th percentile, yields an even more conservative figure of 160.0 mg per day (0.160 g/day).

While no toxicology related issues have been identified at any level of Hayashibara isoamylase consumption, The Hayashibara Company, Ltd. asserts that the figures shown above and in Tables 23 and 24 represent exaggerated intakes, because use of a debranching enzyme would not be appropriate in every starch-containing food

within a given category. Therefore, the actual exposure level may be one to several orders of magnitude less than the very conservative figures presented here.

Table 23
Exposure to Hayashibara Isoamylase
in Appropriate Food Categories

Food Category	Average TOS/g Of Food (mg/g)
Legumes	41.0
Baked Goods	0.023
Non-Alcoholic Beverages	0.043
Milk Products	0.75
Sweets	0.043
Chewing Gum	0.081

Table 24
Estimated Exposure to Hayashibara Isoamylase
in Appropriate Food Categories

Food Category	Intake (g) (CSFII, 1996)	Isoamylase Intake (mg)
Legumes	0.014*	0.0021
Baked Goods	215	4.6
Non-Alcoholic Beverages	82**	15.03
Milk Products	48	61.5
Sweets	22***	1.52
Total		80.0

* Represents a flavoring at 0.05% of a 28 g mean intake

** Not including coffee and tea

** *Including chewing gum

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While the highest treatment level identified for a starchy food by isoamylase was a fried bean flavor for a bean dip (41.0 mg/g), the largest intake of isoamylase modified foods is likely to come from milk based foods (61.5 mg) in the form of reduced-fat dairy products.

As mentioned above, approximately 300 tones of isoamylase have been used by the Hayashibara Company, Ltd. to produce various starch-based sugars over a period stretching for more than two decades. No safety-related issue has been reported to the company, and high-purity maltose made with isoamylase has been in use for intravenous drips [Hayashibara Company, Ltd., Literature, 2000 Vol 3 Tab 26]. The Japanese government has approved isoamylase from various bacterial sources as a food additive [Japanese Food Additive Regulations, 1999 Vol 3 Tab 31].

Therefore, Hayashibara Company, Ltd., submits that its isoamylase enzyme preparation is safe for its intended use as a debranching enzyme in accordance with 21 CFR § 170.3 (o) (9) "Enzymes": Enzymes used to improve food processing and the quality of the finished food.

Table 25
Estimated Intake of Isoamylase in Selected Food Categories

Major Food Codes	Food Category	Use level (mg/g)	Food Intake (g)	Isoamylase Intake (g)	CSFII
	Baked goods, baking mixes				
51	Yeast Breads and Rolls	0.023	50	0.0012	1996
52, 55	Quick breads, pancakes, etc.	0.023	0.1	0.0000023	1996
53	*Cakes, cookies, pastries, pies	0.023	38	0.0009	1996
58	Mixtures mainly grain	0.023	107	0.0025	1996
	Breakfast cereals				
	Ready-to-eat cereals	0	0	0	1996
	Grain products and pastas				
	Rice	0	0	0	1996
	Pasta		0	0	1996
	Snack foods(crackers, chips)		0	0	1996
	Total Vegetables		0		1996
	Fresh vegetables	0	0	0	1996
	Processed vegetables, juices	0	0	0	1996
	Total Fruits		0		1996
	Citrus juices	0	0	0	1996
	Dried fruits	0	0	0	1996
	Non-citrus juices and nectars	0	0	0	1996
	Fruits and mixtures	0	0	0	1996
	Milk products				
114	Yogurt	0.75	8	0.006	1996
131,132, 133, 134	Milk desserts	0.75	24	0.018	1996
140-147	Cheese	0.75	16	0.012	1996
115,116, 118, 119	Milk-Based Beverages	0.75	34	0.0255	1966
	Meat Products				
	Sausages, processed meats		0	0	1996
	Mixtures mainly meat		0	0	1996
	Eggs				
	Egg Products	0	0		1996
	Legumes				
	Legumes	0.15**	0.014**	0.000002	1996
	Nuts and Nut Products				
	Nuts and Nut Products	0	0	0	1996
	Fats and Oils				
	Salad dressings	0	0	0	1996
	Total sugars and sweets				
917,918	Candy (Soft and Hard)	0.043	7	0.0003	1996
911 - 916	Other Sugar Products***	0.081	15	0.00122	1996
	Beverages Non-Alcoholic				
	Coffee	0	0	0	1996
	Teas	0	0	0	1996
See below	Fruit drinks and ades	0.043	82	0.00353	1996
See below	Carbonated drinks	0.043	267	0.0115	1996
	Total Intake (g)			**0.08**	

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Footnotes to Table 25

* Includes dietetic bars
** Represents 0.05% of the average 28 gram per day consumption likely to contain flavoring; usage level reduced to indicate use as flavoring
*** Includes Jams, Jellies, etc.

Food Codes for Beverages

Fruit Drinks and Ades		
	9253	
	9254	
	9257	
	9258	

Carbonated Drinks (Includes most full sugar varieties)

	92410110	
	92410310	
	92410330	
	92410340	
	92410360	
	92410390	
	92410410	
	92410510	
	92410550	
	92410610	
	92410710	
	92410810	
	92411510	
	92432000	
	92433000	

B. Estimation of exposure to other potential migrants from immobilized enzyme preparations

Isoamylase is not immobilized. Therefore, there will be no exposure from migrants.

Conclusion

Isoamylase is a naturally occurring debranching enzyme, which hydrolyzes the α-(1→6) branched glucosidic linkages found in amylopectin and glycogen [Yokobayashi, *et al.*, 1973 Vol 5 Tab 80]. It is secreted by microorganisms, who depend on the enzyme as a part of their strategy to metabolize complex carbohydrates in their environment, such as starch that can then be used as a food source [Norrman *et al.*, 1974 Vol 4 Tab 52].

Likewise, isoamylase is secreted by the human body into fluids responsible for the breakdown of starches and glycogen [Skude, 1975 Vol 4 Tab 59]. Isoamylase has been identified in saliva, pancreatic fluids, serum and secretions of the genital tract [Skude, 2000 Vol 4 Tab 64].

Recently, scientists have discovered that isoamylase plays an important role in the synthesis of amylopectin in important food plants, such as, potato, rice, corn, peas, and barley [Sun *et al.*, 1999 Vol 4 Tab 66]. Therefore, humans have consumed isoamylase or the products of isoamylase since they began consuming grain products.

The Hayashibara Company, Ltd. produces isoamylase under cGMP from *Pseudomonas amyloderamosa*, a naturally occurring, non-pathogenic organism, that shows no growth at 37°C [Harada, *et al.*, 1972 Vol 2 Tab 22]. The enzyme is produced without genetic biotechnologic modification of the organism.

The company has established that *Pseudomonas amyloderamosa* isoamylase has desirable technical effects in foods, and these effects have been widely recognized in patents and the published literature. An estimated exposure level has been calculated, which does not exceed the margin of safety established by several toxicological studies in animals [Lina, 2000 Vol 3 Tab 43]. Further, the enzyme has been shown to be non-mutagenic in a reverse bacterial mutation assay, and in human lymphocytes [Ribera, 2000 Vol 4 Tab 57 and Van Delft, 2000 Vol 5 Tab 76].

Isoamylase is approved as a food additive in Japan, and Hayashibara isoamylase has been used for more than two decades to produce starch-derived sugars and polyols for human consumption. No safety-related instances have been reported over the course of utilizing more than 300 tonnes of the enzyme to produce human food [Hayashibara Company, Ltd. Unpublished Data, 2000 Vol 2 Tab 25 and Fugiwara, 2000 Vol 2 Tab 19].

Based on the data and information available on this enzyme, Hayashibara Company, Ltd. submits that its enzyme preparation is safe for its intended use in foods in general, when used in accordance with current Good Manufacturing Practices. An Expert Panel of scientists qualified by training and experience to evaluate the safety of food ingredients has concurred with this determination. Their opinion is attached to this Notification at Appendix A.

Expert Opinion

Hayashibara Isoamylase Expert Panel Opinion

Introduction

An Expert Panel qualified by scientific training and experience to evaluate the safety of food and food ingredients was assembled to conduct an independent critical evaluation of the safety of Hayashibara Isoamylase as a food ingredient when produced and used in accordance with current Good Manufacturing Practices and meeting the specifications described herein. The Panel members included:

Dr. Joseph F. Borzelleca	Pharmacologist and Toxicologist, Chairman
Dr. Michael Pariza	Director, Food Research Institute, University of Wisconsin
Mr. Cleve Denny	Microbiologist

The qualifications of the members of the Scientific Expert Review Panel (the Panel) are provided in their curricula vitae, which are on file at the offices of Hayashibara International Inc., 8670 Wolff Court, Suite 200, Westminster, Colorado 80030, USA. The Panel's qualifications in their respective scientific fields meet the requirements set forth in the Federal Food, Drug, and Cosmetic Act's definition of generally recognized as safe (GRAS) substances (§ 201(s)) and 21 CFR 170.30(a) "Eligibility for classification as generally recognized as safe (GRAS)".

The Panel was charged by Hayashibara International Inc. to critically evaluate the information and data regarding isoamylase in general and Hayashibara isoamylase specifically. The Panel was then asked to render an opinion, based on scientific principles, on the generally recognized as safe (GRAS) status of Hayashibara isoamylase. The opinion of the Panel is based on the general use of Hayashibara isoamylase in food when manufactured in accordance with U.S. current Good Manufacturing Practices (21 CFR Part 110) and meeting the specifications provided below.

Independently, members of the Panel critically evaluated the results of a literature review on isoamylase, information provided by Hayashibara International, Inc., and other pertinent materials. The Panel conferred by telephone and then met in Washington, D.C. on November 16, 2000 with representatives of Hayashibara International Inc., who reviewed the pertinent safety and functionality information associated with Hayashibara isoamylase. This information was compiled into a GRAS Notification, which supported the eligibility of Hayashibara Isoamylase as a GRAS ingredient in accordance with 21 CFR 170.36. The information provided for the Panel's review was presented in the form stipulated in 21 CFR 170.35.

Conclusion

Based on the Panel's independent and collective critical evaluation of all the information summarized in the GRAS Report, it was determined that Hayashibara isoamylase could be considered generally recognized as safe (GRAS) when produced and used in accordance with cGMP at the levels cited in the Report in the various major food categories as listed by the United States Department of Agriculture in the *Continuing Survey of Food Intakes by Individuals* (CSFII), and, when meeting the specifications described herein.

Signatures

Dr. Joseph F. Borzelleca, Chairman	Date
_____	28 May 2001
Dr. Michael Pariza	Date
_____	May 23, 2001
Mr. Cleve Denny	Date
_____	May 22, 2001

In reaching this conclusion, the Panel relied on the following information: (References refer to those cited in the GRAS Notification)

Chemical Identity and Manufacturing

- Hayashibara isoamylase is produced under current Good Manufacturing Practices

- Final product specifications for Hayashibara isoamylase have been provided. The product meets the specifications shown below:

Hayashibara Isoamylase Specifications

Activity	1,250,000 U/g
Lead	<0.1 ppm
Arsenic	<0.1 ppm
Cadmium	<0.1 ppm
Standard Plate Count	<100 CFU/g
Coliforms	<1
Salmonella sp.	negative/25g

- An analysis of five lots of Hayashibara isoamylase has demonstrated that the products can consistently meet their published specifications

- Chemical and heavy metals analyses of Hayashibara isoamylase products have shown that the products contain no toxicants of concern

- Incoming raw materials for fermentation are food grade

- The source organism for Hayashibara isoamylase, *Pseudomonas amyloderamosa* MU 1174 is:
  - Non-pathogenic
  - Non-GMO
  - A hyperisoamylase-producing strain
  - Derived from *Pseudomonas amyloderamosa* ATCC 21262

- The source organism not a human pathogen, and shows no growth at 37°C

- Seed cultures are checked microscopically for contamination during transfers

- Recent analysis reported a protein content of 19 mg/g of the enzyme preparation

- The enzyme structure has been extensively studied (Katsuya, *et al.,* 1998)
  - Molecular mass of 80 kDa
  - The three-dimensional structure of isoamylase is similar to other enzymes in the α-amylase family, which contain additional domains in the N-terminal region
    - The specific activity of isoamylase may result from an extra domain in the N-terminal region
    - The N-terminal region of isoamylase contains a barrel-type supersecondary structure common to other enzymes in the α-amylase family, and a bound calcium ion, situated in a different position than other enzymes in the alpha-amylase family

Use and Functionality

- The enzyme hydrolyzes 1,6-α-D-glucosidic branch linkages in glycogen, amylopectin, and their α-limit dextrins.

- The enzyme hydrolyzes both inner and outer branching linkages of amylopectin, and is capable of debranching glycogen linkages

- The mode of action of the enzyme may be either endowise on amylopectin or exowise on glycogen

- Hayashibara Isoamylase has a long history of safe use
  - Hayashibara isoamylase is approved as a food additive in Japan
  - It has been used to produce an intravenous drip of high purity maltose
  - An Enzyme Commission number has been assigned

- Several examples of intended uses were provided
  - As a debranching enzyme in:
    - The manufacture of trehalose

- A starch sugar composition
- An enzyme-resistant starch for a reduced-calorie flour replacer
- Starchy products
- A hypocariogenic hydrogenated starch hydrolysate
- A microcrystalline starch base product
- Non-reducing oligosaccharides
- A bean flavor
- A starch emulsifier composition

Safety Studies

- Several safety studies have supported the safe use of Hayashibara isoamylase in food

- An acute study in mice demonstrated that a single dose of *P. amyloderamosa* cells administered intravenously at concentrations up to 4.35 X 10^{10} CFU/kg resulted in no mortalities or adverse clinical signs (ADEC TOX, 2000)

- An acute toxicity study in mice showed no clinically significant findings when the test animals were administered doses of *P. amyloderamosa* via forced oral infusion:
  - ◊ wet cells; LD_{50} was 66.0 g/kg bw
  - ◊ culture filtrate; LD_{50} was >60.0 ml/kg bw
  - ◊ a 40% suspension of the purified enzyme; LD_{50} was >17.0 g/kg bw (Morimoto *et al.*, 1979)

- A sub-chronic 90-day toxicity study in rats showed that the NOAEL was the highest dose tested (228 mg of protein/kg bw/day) (Lina, 2000)

- A bacteria reverse mutation assay showed that the isoamylase test preparation was neither toxic nor mutagenic (TNO, 1999)

- An *in vitro* mammalian chromosome aberration test in cultured human lymphocytes showed that Hayashibara isoamylase did not induce chromosomal aberrations at concentrations up to 5,000 μg/ml (CIT, 2000)

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SUBMISSION END

Submission End

AM

Jones, Kathleen

From: Alan B. Richards [abrichards@hbi-usa.com]
Sent: Thursday, October 18, 2001 4:51 PM
To: Jones, Kathleen
Cc: dexterandassoc@aol.com
Subject: GRN 85 - Isoamylase

Dear Dr. McAveney Jones:

Thank you for your comments. We will review the document in light of these comments and try to have specific answers ready ahead of the teleconference, Thursday October 25, 10:00 am to 12:00 noon EST.

Sincerely,
Alan B. Richards, Ph.D.
Vice President & General Manager
Hayashibara International Inc.
8670 Wolff Court, Suite 200
Westminster, Colorado, USA 80031
Tel: 303-650-4590 Fax: 303-650-9860

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000167

AM

Jones, Kathleen

From: Alan B. Richards [abrichards@hbi-usa.com]
Sent: Tuesday, October 23, 2001 1:19 PM
To: Jones, Kathleen
Cc: dexterandassoc@aol.com; ToxPro@aol.com
Subject: GRN 0085 Conference Call - Isoamylase

Dear FDA Staff and Colleagues:

Thank you for the opportunity to have the conference call concerning GRN 0085 - Isoamylase on Thursday, November 25 at 10:00am (EST). The participants from Hayashibara International Inc. (HBI) will be Dr. Joseph Borzelleca (a member of the Expert Panel on Isoamylase, and Toxicologist), Ms. Lee Dexter (consultant and technical advisor), and myself.

The conference call will be handled through our phone system at HBI. To enter the system:

1. Call 1-303-650-4590 (HBI's main telephone number)
2. When prompted for the extension enter 290.
3. When prompted for the password enter 102501 (the date).

If you have any problems getting into the conference call center, please call the main telephone number (303-650-4590) and enter extension 201, which is the secretary's. She can transfer your call to the conference call center.

Sincerely,
Alan B. Richards, Ph.D.
Vice President & General Manager
Hayashibara International Inc.
8670 Wolff Court, Suite 200
Westminster, Colorado, USA 80031
Tel: 303-650-4590 Fax: 303-650-9860

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000168

AM

Jones, Kathleen

From: Alan B. Richards [abrichards@hbi-usa.com]
Sent: Thursday, October 25, 2001 12:23 PM
To: Jones, Kathleen
Cc: Kahl, Linda S
Subject: GRN 0085 - Teleconference

Dear Dr. McAveney Jones:

I would like to thank you and your colleagues for taking the time and energy to discuss your comments on the GRAS Notice with us. As we reviewed the document in light of the comments we could understand the points made and questions that arose.

The solution of an amendment addressing each of the issues that were raised is very appreciated.

Finally, I personally appreciated the general comments about our submissions and our presentations to CFSAN. HBI will make every effort to make future submission in line with these comments.

We will have the amendment to you within two weeks, and I will call to make sure it has gotten to your office.

Sincerely,
Alan B. Richards, Ph.D.
Vice President & General Manager
Hayashibara International Inc.
8670 Wolff Court, Suite 200
Westminster, Colorado, USA 80031
Tel: 303-650-4590 Fax: 303-650-9860

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000174

AM

Jones, Kathleen

From: Alan B. Richards [abrichards@hbi-usa.com]
Sent: Friday, October 26, 2001 4:48 PM
To: Jones, Kathleen
Subject: GRN 0085 - Isoamylase

Dear Dr. McAveney Jones:

At yesterday's meeting, one of the reviewers made comments concerning antibiotic and microbiologic issues. The male (I am sorry but we could not hear distinctly some of the names of the individuals) reviewer mentioned about a Document on the Web Page that addressed the data necessary when a microbiologic organism is used to produce a final product (antibiotic production, etc.).

We have been searching for such a document but have not been able to locate or perhaps recognize it. Can you provide me with the name of the document, or the email address or phone number of the reviewer.

Sorry to trouble you and thanks for any assistance you can give.

Sincerely,
Alan B. Richards, Ph.D.
Vice President & General Manager
Hayashibara International Inc.
8670 Wolff Court, Suite 200
Westminster, Colorado, USA 80031
Tel: 303-650-4590 Fax: 303-650-9860

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000175

AM

Jones, Kathleen

From: Alan B. Richards [abrichards@hbi-usa.com]
Sent: Thursday, November 01, 2001 9:46 AM
To: Jones, Kathleen
Subject: GRN 0085 - Isoamylase

Dear Dr. McAveney Jones:

Thank you for going to the trouble of providing this information. As you mentioned, we will review and consider it as recommendations from the Agency responsible for reviewing our submissions.

Sincerely,
Alan B. Richards, Ph.D.
Vice President & General Manager
Hayashibara International Inc.
8670 Wolff Court, Suite 200
Westminster, Colorado, USA 80031
Tel: 303-650-4590 Fax: 303-650-9860

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000177

HAYASHIBARA INTERNATIONAL INC.
8670 Wolff Court, Suite 200
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AM

November 19, 2001

Kathleen McAveney Jones, Ph.D.
U.S. Food and Drug Administration
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
Division of Biotech and GRAS Notice Review, HFS-206
200 "C" Street, SW
Washington, DC 20204

RE: GRN 0085 (Isoamylase): Response to Review Team's Questions

Dear Dr. McAveney Jones:

This submission is in response to questions raised by the CFSAN Review Team regarding information presented in GRN 0085 on Hayashibara isoamylase. A written copy of the questions was sent from your office to Hayashibara International Inc. on October 18, 2001. A follow-up telephone conference call with the review committee was held on October 25, 2001 at which time the questions were verbally explained and responses were provided. This present submission is designed to formally respond to the questions.

The responses are in the same order as they were presented in your written communication of October 18, 2000. In our conference call it was decided that items 12 and 13 did not need a written response.

We hope that this submission adequately addresses the questions asked. If they do not, we would be pleased to offer additional information.

Sincerely,

(b)(6)

Alan B. Richards, Ph.D.
Vice President & General Manager

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HAYASHIBARA GROUP

Responses to GRAS Review Team for Hayashibara Isoamylase (GRN 0085)

This document contains written responses to questions of GRAS Review Team (GRN 0085; Hayashibara isoamylase) that were provided in writing to the Sponsor on October 18, 2001, and discussed during a teleconference October 25, 2001. The original documents for the references in [brackets] are located in the original GRAS Notice submission (GRN 0085). The responses are listed in the same order as provided in the original communication (October 18, 2001).

1. Intended Use

This section provides general categories and specific examples of the intended use of Hayashibara isoamylase. By intended use, the Sponsor means the food products in which isoamylase will be a direct food additive.

Isoamylase is a debranching enzyme that hydrolyzes α-1,6 bonds that link one chain of glucose molecules (amylose) to a second chain. The debranching activity can be used alone or in concert with other enzymatic or chemical reactions to produce a number of products. The products can be divided into three categories:

a. Isoamylase may be used, usually with other enzymes, as a processing aid to hydrolyze starch and produce food ingredients, such as glucose, maltose, trehalose, high fructose corn syrup, maltotetraose, dextrins, and oligosaccharides [Yokobayashi, *et al.*,1970 Vol 5 Tab 79, Yokobayashi, *et al.*,1973 Vol 5 Tab 80 and Sun *et al.*, 1999 Vol 4 Tab 66]. The enzyme is usually degraded and removed during the production process.

b. Isoamylase may be used to partially debranch starches, which modifies their properties. These modifications have been developed and used over many years, and are optimized for use in specific food categories. Modified starches, therefore are food ingredients that can provide specific functions in food preparations. Isoamylase has recently been approved as a food additive for use in the modification of food starches [*Federal Register* Vol 66, No. 63, April 2, 2001 Vol 2 Tab 17]. The enzyme may or may not be degraded during the production process.

c. Isoamylase may be added directly to starch-based food formulations to modify the quality, caloric value or properties of the final food product [US Patent 6,090,594 Vol 4 Tab 70]. The enzyme is not likely removed from the final product, and may or may not be degraded in the manufacturing process.

Examples of the intended use of Hayashibara isoamylase in various products are presented in the following tables. The range of uses of these potential products includes several major food categories, such as baked goods, milk products, sweets, and non-alcoholic beverages.

1a. Examples of the Intended Use of Isoamylase to Produce Starch-derived Sugars

Product	Description
Trehalose	Isoamylase used with other enzymes to hydrolyze starch, producing a unique multifunctional disaccharide.
Hypocariogenic hydrogenated starch hydrolysate	Starch is partially debranched with isoamylase resulting in a hydrolysate that can be used to sweeten various products, but is hypocariogenic.
Non-reducing oligosaccharides	Produced by the debranching of starch with isoamylase.

1b. Examples of the Intended Use of Isoamylase to Produce Novel Starch-based Ingredients

Product	Description
Enzyme–resistant starch for reduced-calorie flour replacer	Isoamylase is used with other methods to modify starch, producing flour that is resistant to natural enzymatic hydrolysis.
Microcrystalline starch-based product	Isoamylase is used to debranch starch and then the product is milled to microcrystalline particle size. Has reduced calories.

1.c Examples of the Intended Use of Isoamylase to Produce Starch Food Products

Product	Description
Bean flavor	Bean starch is debranched with isoamylase and treated with other methods to produce a fried bean flavor.
Starch emulsifier	Isoamylase debranches starch, which is further modified. Product can be used as an emulsifier in a variety of specialty processed dairy products.

2. Technical Effects

The technical effect of isoamylase is a debranching enzyme that hydrolyzes α-1,6 bonds in starch and similar molecules. Isoamylase can be used to produce starch-derived sugars or oligosaccharides, to produce modified starch ingredients, and to directly modify the textural and organoleptic properties of starch-containing foods.

The controlled enzymatic hydrolysis of starch can result in products with a wide range of molecular weights. The types of hydrolytic fractions produced are dependent on several factors, including the substrate concentration, the temperature of the reaction, the reaction time, and the presence of co-reactants [Whistler, *et al.,* 1997 Vol 5 Tab 84]. The chemical and enzymatic modification of starch has been studied for decades, and food scientists have developed specific reaction sequences to produce desired effects in foods. Since starch is relatively inexpensive as a raw material, the reaction sequences necessary to produce a desired effect are often considered proprietary or are protected by patents.

The products listed in the tables below represent the end result of the treatment (debranching) of starch with isoamylase. The specific technical effects, of the various debranching procedures, are given in table below.

For starch-based sugars, such as trehalose, starch hydrolysate, and non-reducing oligosaccharide, isoamylase is used to debranch amylopectin into smaller molecular fragments. The larger fragments can be precipitated as starch hydrolysate, or specific oligosaccharide fragments can be created. In the case of trehalose production, isoamylase hydrolyzes (debranches) amylopectin, allowing other carbohydrases to more completely hydrolyze the starch to the appropriate disaccharide. This dramatically increases the yield of trehalose.

To prepare modified starch ingredients, longer reaction times are generally utilized to fragment the starch molecule into very small or very hard fractions [Whistler, *et al.,* 1997 Vol 5 Tab 84]. This principle is often used to create starch-based ingredients with many of the properties of fat, or with reduced reactivity to natural digestive enzymes (resistant starch). These products often are formulated into low-calorie, low-fat, or no-fat food items. Examples include the microcrystalline starch, and resistant starch described below.

Alternatively, the use of isoamylase during food preparation can directly affect the texture and organoleptic properties of foods. Modification of starch properties can affect its ability to combine with lipids, as in the bean flavor and starch emulsifier combination.

The tables are provided to show examples of the physical or functional technical effects that can be occur using isoamylase. Each of the examples is from issued U.S. patents.

2a. Technical Effects of Isoamylase in the Production of Starch-derived Sugars

Product	Specific Technical Effect
Trehalose	Used as a debranching enzyme to enhance the efficiency of trehalose producing enzymes to amylopectin substrates
Hypocariogenic hydrogenated starch hydrolysate	Used to hydrolyze amylopectin. Polysaccharides that are difficult to digest are obtained.
Non-reducing oligosaccharides	Used to convert amylopectin to non-reducing oligosaccharide. Used to accumulate maltosyl maltoside, maltotriosyl maltoside, and maltotriosyl maltotrioside.

2b. Technical Effects of Isoamylase to Produce Novel Starch-based Ingredients

Product	Specific Technical Effect
Enzyme–resistant starch for reduced-calorie flour replacer	Used to produce a high-melting precipitate from amylopectin, and to increase the yield and resiliency under baking conditions of the resistant starch.
Microcrystalline starch-based product	Used to hydrolyze amylopectin to produce short chain glucooligosaccharides with a particle size of ~10 microns
Resistant Starch	Used to produce a high proportion (28.9% vs. 7.3% in a maltodextrin) of resistant starch structures from an aqueous slurry of gelatinized starch.

2.c Technical Effects of Isoamylase in the Production of Directly Modified Starch-based Food Products

Product	Specific Functional Effect
Bean flavor	Used as a carbohydrase to liberate reducing sugars from bean starch, which are combined with lipids.
Starch emulsifier	Used to create low-molecular weight fractions of starch for combination with glycerol monostearate.

3. Use Levels/Exposure

Based on the intended use of Hayashibara isoamylase, and the concentrations needed to provide the appropriate technical effect, it was possible to calculate the Total Organic Solids (TOS) contribution of an isoamylase preparation in a given food product. The

TOS value used for the calculations was taken from the mean of five lots as shown on page 31 of GRN 0085. Tables showing the TOS in representative food products are provided.

3a. Examples of the Concentration of Hayashibara Isoamylase TOS in Starch-derived Sugars

Product	TOS/g
Trehalose	~0.0454mg/g; [GRN 0045]
Hypocariogenic hydrogenated starch hydrolysate	~0.081mg/g; [US Patent 5,493,014]
Non-reducing oligosaccharides	~0.0015mg/g; [US Patent 5,780,620]

3b. Examples of the Concentration of Hayashibara Isoamylase TOS in Novel Starch-based Ingredients

Product	Description
Enzyme–resistant starch for reduced-calorie flour replacer	~0.0687mg/g; [US Patent 5,962,047]
Microcrystalline starch-based product	~0.041mg/g; [US Patent 6,090,594]
Enzyme–Resistant Starch for Reduced-Calorie Flour Replacer	~0.0016mg/g; [US Patent 6,013,299]

3c. Examples of the Concentration of Hayashibara Isoamylase TOS in Starch Food Products

Product	Description
Bean flavor	~41.0mg/g; [US Patent 5,773,055]
Starch emulsifier	~0.81mg/g; [US Patent 6,017,388]

Using these examples, it was also possible to calculate the potential concentration of Hayashibara isoamylase in several major food categories. The results are shown in the table 3d. As an example, the average intake of Hayashibara isoamylase in baked goods is anticipated to equal approximately 0.023mg/g of food. The most concentrated use of isoamylase identified was in the modification of a legume paste (41.0mg/g of food) to create an oil-soluble high-strength flavor [US Patent 5,773,055 Vol 4 Tab 67]. The highest daily exposure to isoamylase may potentially come from consumption of reduced-fat milk products (0.75mg/g of food), such as ice creams and yogurts. However, in this case it is anticipated that the heat of processing or pasteurization would inactivate essentially all of the enzyme activity.

3d. Average Total Organic Solids Contributed by Hayashibara Isoamylase in Various Food Categories

Food Categories	Average TOS/g of Food (mg/g)
Baked Goods	0.023
Legumes	41.0
Milk products	0.75
Sweets	0.043
Chewing Gum	0.081

Isoamylase is also used as a processing aid in the production of starch-derived sugars. However, during commercial starch hydrolysis, the enzyme is inactivated and removed as part of the purification of the final product. As an example, approximately 0.0454mg of isoamylase preparation (≥ 1,250,000U/g) is used in the production of 1.0 gram of trehalose. Essentially all of the enzyme is inactivated and removed in the course of processing [GRAS Notice No. 0045, 2000 Vol 5 Tab 83]. The table below illustrates that the average residual isoamylase concentration (by ELISA) of three lots of commercial trehalose is below the level of detection.

3.e Residual Isoamylase Content of Hayashibara Trehalose

Immunogen	Lot No	ELISA Detection Range	Analytical Results
Isoamylase	60911, 61011 and 61018	50-1,000ng/ml	<50ng/ml

In the telephone conference with CFSAN personnel, the fact that enzymes are typically removed during the commercial processing of sweeteners was discussed. Therefore, CFSAN advised that the intended use of isoamylase to produce starch-based sugars did not actually contribute to isoamylase exposure.

The estimated intake of Hayashibara isoamylase is shown in table 3f. (Table 25 of GRN 0085) was revised to indicate that consumers would not be exposed to the enzyme preparation when consuming products rich in starch-based sweeteners, such as high fructose corn syrup. This adjustment particularly affected the carbonated drink sub-category within the major Beverages, Non-Alcoholic category. As a result, the estimated mean intake of Hayashibara isoamylase was reduced to 71.0mg/person/day. This value represents the total estimated mean intake of isoamylase TOS per person per day. An equation to determine the intake of TOS per food category was presented in Section VIII (Exposure), page 97 of GRN 0045.

Table 3f. Estimated Intake of Isoamylase in Selected Food Categories
From Table 25 - GRN 0085 - Based on the *Continuing Survey of Food Intakes by Individuals* (1996)

Major Food Codes	Food Category	Enzyme Use Level (mg TOS/g of food)	Food Intake (g)	Isoamylase Intake (g)
	Baked goods, baking mixes			
51	Yeast Breads and Rolls	0.023	50	0.0012
52, 55	Quick breads, pancakes, etc.	0.023	0.1	0.0000023
53	*Cakes, cookies, pastries, pies	0.023	38	0.0009
58	Mixtures mainly grain	0.023	107	0.0025
	Breakfast cereals			
	Ready-to-eat cereals	0	0	0
	Grain products and pastas			
	Rice	0	0	0
	Pasta		0	0
	Snack foods(crackers, chips)		0	0
	Total Vegetables			
	Fresh vegetables	0	0	0
	Processed vegetables, juices	0	0	0
	Total Fruits			
	Citrus juices	0	0	0
	Dried fruits	0	0	0
	Non-citrus juices and nectars	0	0	0
	Fruits and mixtures	0	0	0
	Milk products			
114	Yogurt	0.75	8	0.006
131,132, 133, 134	Milk desserts	0.75	24	0.018
140-147	Cheese	0.75	16	0.012
115,116, 118, 119	Milk-Based Beverages	0.75	34	0.0255
	Meat Products			
	Sausages, processed meats		0	0
	Mixtures mainly meat		0	0
	Eggs			
	Egg Products	0	0	
	Legumes			
	Legumes	0.15**	0.014**	0.000002
	Nuts and Nut Products			
	Nuts and Nut Products	0	0	0
	Fats and Oils			
	Salad dressings	0	0	0
	Total sugars and sweets			
917,918	Candy (Soft and Hard)	0.043	7	0.0003
911-916	Other Sugar Products***	0.081	15	0.00122
	Beverages Non-Alcoholic			
	Coffee	0	0	0
	Teas	0	0	0
9253,9254,9257,9258	Fruit drinks and ades	0.043	82	0.00353
	Carbonated drinks	0	267	0
	Total Intake (g)			**0.071**

* Includes dietetic bars. ** Represents 0.05% of the average 28 gram per day consumption likely to contain flavoring; use level reduced to indicate use as flavoring contain flavoring; usage level reduced to indicate use as flavoring *** Includes Jams, Jellies, etc.

However, even an intake of 71.0mg/person/day is considered unlikely. To reach this level of consumption, the average consumer would have to consume foods (in a single day) from every category that could contain isoamylase, and isoamylase would have to be an ingredient in all the foods within those categories. Since Hayashibara isoamylase will only be used for specific technical effects, a 100% market penetration of all relevant food categories is not realistic.

As an example, if 10% of the population of Japan consumed all of the isoamylase that was produced by Hayashibara in 1999 (the year of highest sales), then each person would actually consume 9.4mg/day. This amount may more accurately represent daily consumption, as they would actually exist in a mature market.

4. Enzyme Doses Used in Various Studies

The question concerned the various units used to describe the doses in the safety studies that were provided.

None of the lots used in the safety studies were assayed for total organic solids (TOS). All studies performed, other than the three studies in Japan in 1979, were standardized to enzymatic units per ml. However, in three studies the doses can be equated to TOS. Hayashibara assayed 5 lots of commercial enzyme and calculated the mean TOS concentration (4.05%; page 31 of GRN 0085).

The most relevant study to demonstrating the safe consumption of Hayashibara isoamylase is the 90-day (13-week) subchronic oral toxicity study in rats. The other two studies in which a TOS concentration can be calculated are a bacterial reverse mutation study, and an *in vitro* human lymphocyte chromosomal aberration assay. In these latter two studies, a TOS measurement is not directly relevant.

In the subchronic study the specific gravity of the preparation tested was 1.20kg/L (1.2g/ml), therefore the TOS is equal to 48.6mg TOS/ml (TOS=4.05%). The NOAEL, which was the highest dose established by the investigators, was 10ml/kg bw/day. Therefore, the daily intake of isoamylase TOS was approximately 486mg/kg bw/day. Using very conservative assumptions, it is estimated that the maximum potential mean intake of isoamylase TOS is approximately 71mg/person/day (See Response 3).

Therefore, the margin of safety in an adult human would be greater than 400 fold. The TOS of the three studies is given below in table 4a.

4a. Highest Dose of TOS Administered in Three Studies

Study	Date	Lot #	Activity	TOS and Concentration	Specific gravity
Subchronic Oral Toxicity in Rats #V99.646	01-00	80519	1,362,500 U/g solution	**TOS = 486mg/ml or 486mg/kg bw/day at the NOAEL (highest dose)** 19 mg protein/g solution	1.20kg/L
Bacterial Reverse Mutation #V99.526	16-06-99	81114 and 90308	1,367,500 and 1,375,000U/g of solution	**TOS = 44.5mg/ml** 19mg protein/g solution	1.01 and 1.02kg/L
In Vitro Human Lymphocyte Chromosomal Aberration #20147 MLH	12-01-01	00426	1,350,000 U/g of solution	**TOS = 44.1mg/ml** 19mg protein/g solution	1.188 kg/L

Two of the remaining studies assess the pathogenicity and toxigenicity of the production bacteria when administered once via the oral, or intravenous routes. Therefore, the TOS of Hayashibara isoamylase preparation is not applicable to those studies. One of these two studies was performed in Japan in 1979, where the dose was given in mg of the wet bacterial cells per kg of animal body weight. This was done at, and according to the procedure of the Japan Food Research Laboratories, Tokyo, Japan. The LD_{50} of the study was greater than 66,000mg/kg body weight. The second study was performed in 2000 by a certified laboratory in France according to the protocol described in "Development of Assessment Methods for Safety of Animal Feed and Microbial Feed" published by the Ministry of Agriculture, Forestry and Fisheries of Japan. This protocol called for doses to be given intravenously in colony forming units (CFU)/kg body weight. After 14 days the highest dose (4.35×10^{10} CFU/kg) did not result in any toxic changes or proliferation of the organism. While the two doses are not comparable, the Sponsor agrees with members of the Expert Panel that the studies demonstrate that the strains of *Pseudomonas amyloderamosa* used to produce commercial lots of Hayashibara isoamylase are neither pathogenic nor toxigenic to the test animals.

4b. Highest Doses of Bacterial Cell Inoculation in Two Studies

Study	Date	Lot #	Activity	Concentration	Specific gravity
Pathogenicity in Mice #Tg321	19-12-00	Bacterial strain MU-1147 (Commercial)	Freshly cultured cells	Highest dose 4.35×10^{10} CFU/kg (8.7×10^{9} CFU/ml) 5ml/kg	N/A
Acute Toxicity in Mice 12110175-1	28-12-79	Unknown	Bacterial cells (wet)	Highest dose 66,000 mg/kg (60.0ml/kg)	N/A

The final two acute studies were performed in 1979 by the Japan Food Research Laboratories, Tokyo, Japan. Hayashibara personnel in Japan have stated that the samples were produced by a production strain of the bacteria, since these studies were used for regulatory approval. The studies used culture filtrate and a concentrated suspension of isoamylase prepared from a lyophilized enzyme sample. The doses were provided in mg of sample per kg body weight. The TOS, mg protein, or enzymatic units are not known. Twenty mice (10 female, 10 male) were used per treatment group for each study. The oral LD_{50} of the studies were greater than 60,000mg/kg body weight, and approximately 17,000mg/kg body weight, respectively. Deaths and clinical symptoms were reported in the latter study. These appeared to be related to the concentrated nature of the test sample (40% suspension of enzyme). Although the TOS concentration and enzymatic units are unknown, it is felt that the amount of enzyme and other organic solids represented in these two studies provides a measure of assurance as to the safety of preparations of Hayashibara isoamylase.

4c. Highest Doses of Culture Filtrate and Enzyme in Two Studies

Study	Date	Lot #	Activity	Concentration	Specific gravity
Acute Toxicity in Mice 12110175-2	28-12-79	Unk[1]	Culture filtrate	Highest dose 60,000 mg/kg (60.0ml/kg)	Unk[1]
Acute Toxicity of Isoamylase in Mice 12110175-3	28-12-79	Unk[1]	Isoamylase	40% suspension of Isoamylase	Unk[1]

[1]Unk = Unknown

Although it would have been optimal to have all doses standardized in specific units, such as TOS, this was not possible.

In addition to the animal studies there is a history of safe use in Japan of over 20 years. During this time no complaints have been registered relative to the safety of the enzyme, and the Hayashibara personnel who produce and use the enzyme each day

have never reported clinical complaints to company health officials that were associated with exposure to Hayashibara isoamylase.

The Sponsor concurs with the opinion of the Expert Panel that the available information supports the safety and GRAS status of Hayashibara isoamylase for its intended use under cGMP.

5. 90-Day Subchronic Rat Toxicity Study

A re-review of the raw data from the 90-day (13-week) subchronic rat toxicity study was performed by Dr. Joseph F. Borzelleca (Isoamylase Expert Panel). The purpose was to clarify the cause of death of six (6) rats who died while being fed Hayashibara isoamylase at concentrations of up to 10% of the diet. Dr. Borzelleca's findings are attached. In brief, it was concluded that 4 of the six deaths were due to gavage failure. The remaining 2 deaths could not be determined with certainty because one animal had been cannibalized and the other was decomposed. However, it appears likely that the 2 deaths were not associated with isoamylase because no other consistent treatment-related, dose-dependent effects were observed in any of the other variables studied.

Pages 000190-000191 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

6. Genetic Stability

Hayashibara International Inc. would like to clarify the manner in which it assures the genetic stability of its isoamylase producing strain of *Pseudomonas amyloderamosa.* In GRN 0085, the sponsor described its procedure for assuring the cultural purity of the organism on page 42. The description focused on the routine use of microscopic examination of a seed culture to determine purity. A statement was also made, that at the present time Hayashibara does not analyze for genetic stability. This statement was meant to indicate that the company does not perform DNA analysis, nor does it do a complete biochemical analysis of the organism during production runs.

When the freeze-dried production stock is low, the organism is propagated, and tested for isoamylase production, morphology, and a complete biochemical panel is performed for identification.

Dr. Michael Pariza, one of the members of the Isoamylase Expert Panel, has indicated that the company is in fact indirectly measuring the genetic stability of the production strain through its ongoing testing of enzyme production. Dr. Pariza indicated that any loss in enzyme producing ability would indicate a change in genetic stability.

7. Bacterial Contamination

During production of Hayashibara isoamylase the source organism, *Pseudomonas amyloderamosa*, is propagated in several steps. The organism is microscopically examined at each step to detect bacterial contamination. If contamination is detected, the critical control procedure requires that the batch be destroyed. The batch is not reworked.

8. Production Strain and Commercial Isoamylase Lots Used

Several animal studies were reported in GRN 0085 in which either *Pseudomonas amyloderamosa* or Hayashibara isoamylase was used to test for the pathogenic, toxigenic or mutagenic potential of the organism and enzyme.

Three acute studies were performed in Japan in 1979. They included oral treatment of female and male mice with the source organism, *Pseudomonas amyloderamosa*,

culture filtrate, and concentrated (40%) isoamylase. Study reports are available; however, no explanation of the nature of the bacteria or enzyme preparation is given. From contact with Hayashibara Company, Ltd., who commissioned these studies, it was noted that the studies were performed for the purpose of getting regulatory approval of isoamylase in Japan. Therefore, it is the company's position that the samples were from the production organism and commercial-type lots of the enzyme. It is the Sponsor's understanding that the Japanese authorities would have required the use of production samples.

All other studies were performed using the production strain of *Pseudomonas amyloderamosa* or a commercial production lot of Hayashibara isoamylase. Certificates of Analysis are available for all lots used in these safety studies.

9. Naturally Occurring Organism

The CFSAN reviewers took exception to the statement made in GRN 0085 on pages 67 and 102 that the Hayashibara isoamylase is produced by *Pseudomonas amyloderamosa*, which is a naturally occurring, non-pathogenic, non-toxigenic organism. This suggested that the production strain is naturally occurring, which is not correct. As discussed during the telephone conference, the description on page 2 of GRN 0085 is clearer. The parental strain from which the production strain was derived is naturally occurring, secretes isoamylase extracellularly, and is non-pathogenic and non-toxigenic. The production strain was derived from the parental strain using classical NTG-mutation techniques.

Dr. Michael Pariza was asked for a response to the issue of whether the production strain for Hayashibara isoamylase was a naturally occurring organism. Dr. Pariza indicated that one could say that the parental strain of *Pseudomonas amyloderamosa* was a naturally occurring organism. However, one could not say with certainty whether the current production strain for Hayashibara isoamylase occurred in nature or not.

Dr. Pariza indicated that NTG-mutation was a long-standing, well-accepted method for enhancing productivity. He also stated that random mutations are known to delete old traits, but not to add new traits, such as the production of a toxin (Pariza, *et al.,* 2000). Therefore, in his opinion, it is highly unlikely that the organism has or would become toxigenic.

10. Antibiotic Production

Three lots of Hayashibara isoamylase were tested for antimicrobial activity. All three samples were from commercial lots of enzyme. The assays were preformed at the Institut Européen de l'Environnement de Bordeaux, Bordeaux, France. This is a laboratory that performs official assays for the Ministry of Health, the Ministry of the Environment and other governmental agencies. This study was performed according to the U.N. Food and Agriculture Organization protocol using bacterial strains recommended for the antibiotic assay.

Using this test system, no antimicrobial activity was detected.

10. Antibacterial Activities in Isoamylase Preparations

Bacteria	ATCC[a] number	Antibacterial activities in Isoamylase preparations (µg/g)[b]		
		lot n°91022 10.22.1999	lot n°91119 11.26.1999	lot n°00218 02.18.2000
Bacillus subtilis	6633	negative	negative	negative
Bacillus cereus	9634	negative	negative	negative
Enterococcus faecalis	8043	negative	negative	negative
Escherichia coli	8789	negative	negative	negative
Serratia marcescens	14756	negative	negative	negative
Staphylococcus aureus	6538	negative	negative	negative

a: American Type Culture Collection. b: µg of antibiotic per gram sample.

11. *Food Chemicals Codex*

In Section III of GRN 0085, the statement is made that Hayashibara isoamylase is in compliance with the general specifications for enzymes published in the *Food Chemicals Codex*, [*Food Chemicals Codex*, 1996 Vol 5 Tab 82]. For clarification the 1996 edition of the *Food Chemicals Codex* is the 4th edition. Therefore, the Sponsor would like to amend the reference to compliance with the *Food Chemicals Codex*, by including reference to the most current edition.

AM

Hayashibara International Inc.

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303-650-4590 (tel) – 303-650-9860 (fax)

Facsimile Transmission

To: **Kathleen McAveney Jones, Ph.D.**
Center for Food Safety
and Applied Nutrition
Office of Food Additive Safety
Division of Biotech and FRAS Notice Review, HFS-206
5100 Paint Branch Parkway
College Park, MD 20740

Date: February 12, 2002
Phone: 202-418-3097
Fax: 202-418-3131
Total pgs. 24

From: Alan B. Richards Ph.D.
Hayashibara International Inc.
8670 Wolff Ct., Ste. 200
Westminster, CO 80031

BEST ORIGINAL COPY

RE: GRN 0085
Response to a Request for Additional Information

Dear Dr. Jones:

Please find attached the journal articles that you requested in our telephone conversation at approximately 9:30 a.m. on February 12, 2002.

We would be pleased to supply additional information if needed. Please contact me directly.

Sincerely,

(b)(6)

Alan B. Richards, Ph.D.

If there is a problem with this transmission, please call 303-650-4590

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AM

Jones, Kathleen

From: Alan B. Richards [abrichards@hbi-usa.com]
Sent: Wednesday, February 13, 2002 10:00 AM
To: Jones, Kathleen
Subject: RE: new mail address

Dr. Jones,
Did you receive all the pages of the 4 articles? We placed the sensitivity on fine, but are worried as to whether they are good enough copies.

Unfortunately, because of binding of the journal, the copy of the Lui paper was the best we could do.

Regards,
Alan B. Richards
-----Original Message-----
From: Jones, Kathleen [mailto:Kathleen.Jones@cfsan.fda.gov]
Sent: Tuesday, February 12, 2002 9:07 AM
To: Alan B. Richards (E-mail)
Subject: new mail address

Dr. Richards,

Below is our current correct mail address.

Kathleen Jones

Kathleen McAveney Jones, Ph.D.
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000219

Reference List for Industry Submission, GRN 000085

Pages	*Author*	*Title*	*Publish Date*	*Source*	*BIB_Info*
000190-000191	NA	A Critical Evaluation of the Sub-chronic (13 weeks) Oral Toxicity Study With Isoamylase in Rats	NA	TNO Report	Volume 99.646
000196-000201	Harada, Tokuya, Yokobayashi, Kozi; Misaki, Akira	Formation of Isoamylase by Pseudomonas	October 1968	Applied Microbiology	pgs 1439-1444
000202-000209	Norrman, Jonas; Wober, Gunter	Comparative Biochemistry of a-Glucan-Utilization in Pseudomonas amyloderamosa and Pseudomonas saccharophila - Physiological Significance of Variations in the Pathways	175	Arch. Microbial.	Volume 102, pgs 253-260
000210-000215	Lai, J.; Liu, H.	Production enhancement of Pseudomonas amyloderamosa isoamylase	1996	Bioprocess Engineering	Volume 15, Number 3, pgs 139-144
000216-000218	Wu, Dong-Her; Wen, Chiou-Yen; Lin, Long-Liu; Chu, Wen-Shen; Hsu, Wen-Hwel	Effect of pH on isoamylase production by Pseudomonas amyloderamosa WU 5315	1994	Letters in Applied Microbiology	Volume 19, pgs 67-69

NA- Not applicable