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TREHALOSE
produced by a novel enzymatic process

Dossier prepared and submitted on behalf of
Hayashibara Co., Ltd., 2-3 Shimoishii 1-chome, Okayama 700,
Japan for evaluation pursuant to the EU Novel Foods Regulation
(258/97) by the UK Advisory Committee on
Novel Foods and Processes

Under the "UK Novel Foods and Novel Food Ingredients (Amendment) Regulations 1999" and the corresponding "Guidelines on the disclosure of information and confidentiality in respect of applications made to the ACNFP", this dossier and the pertinent parts of the pivotal safety studies are to be made public. Certain information related to marketing and the production process is confidential and has been removed from this version. However, it is contained in the original dossier and has been given to the Committee for their consideration.

The animal studies which were commissioned by the applicant in order to fulfill the toxicological requirements, were conducted with the approval and under the supervision of the competent Animal Welfare Committee.

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1. Introduction

Trehalose is a naturally occurring disaccharide which consists of two glucose molecules linked in a 1,1-position by an α-glycosidic bond (Figure 1)[1]. It is produced in bacteria and yeast cells, fungi, algae, and a few higher plants. In many insects it is produced as a major blood sugar and as a reserve carbohydrate during periods of dehydration and freezing. Trehalose also occurs at low concentrations in a number of foods which are consumed as part of a regular diet (mushrooms, bread, fermented beverages, honey).

The Advisory Committee on Novel Foods and Processes (ACNFP) assessed the safety of trehalose, extracted from yeast, in 1990. The intended food applications that were considered by the Committee at that time, included the use of trehalose for the stabilization of certain foodstuffs during the drying process and upon rehydration (e.g., milk powder, dry soups). On advice of the ACNFP, the Committee on Toxicity (COT), and the Food Advisory Committee (FAC), trehalose was accepted for use in foods (except infant formulae and follow-on formulae) in April 1991 (MAFF, 1991; ACNFP, 1990, 1991).

Hayashibara Co., Ltd., Okayama (Japan) has developed a novel enzymatic production process. In this process, liquefied starch serves as raw material. Four enzymes which have not hitherto been used in food or food manufacturing in the EU, are applied in this process. The two pivotal enzymes are produced by a strain of *Arthrobacter*

[1] Synonyms: α,α-trehalose, α-D-trehalose, D-(+)–trehalose, α-trehalose, D-trehalose, mycose ("mushrooms sugar"). Brand names: Trehaose™, Treha™ (Hayashibara Co., Ltd.)

ramosus. One enzyme converts the terminal (reducing) maltosyl unit of maltooligosaccharides (DP ≥3) to a trehalose unit. The other enzyme hydrolyzes the α-1,4 glycosidic bond adjacent to the trehalose unit thereby liberating trehalose (Figure 2)[2]. In order to increase the yield of the process, isoamylase from *Pseudomonas amyloderamosa* and cyclodextrin glucosyltransferase (CGTase) from *Bacillus stearothermophilus* are used as ancillary enzymes. None of the source microorganisms of these enzymes is genetically modified.

The present application for authorization of trehalose produced by a novel process was prepared according to the EU Commission's guidelines on the scientific information necessary to support applications for placing on the market of novel foods or novel food ingredients (Commission Recommendation (97/618/EC) and the Guidance Notes on Novel Foods and Novel Food Ingredients Legislation of the UK Ministry of Agriculture, Fisheries and Food (June 1999). Trehalose was identified as belonging to class 6 (foods produced using a novel process. Using the ACNFP's Computerized Decision Tree package, the data requirement was identified. The outcome of this evaluation is shown in Annex 1.

For evaluation of a class 6 product the following information is required (numbering according to Commission Guideline, Table II):

I. Specification of the Novel Food (NF)

II. Effect of the production process applied to the NF

III. History of the organism used as the source of the NF

IX. Anticipated intake/extent of use of the NF

[2] The biosynthesis of trehalose in *Rhizobia* and certain other microorganisms relies on the same enzymatic steps (Kato, 1999; Streeter & Bhagwat, 1999).

X. Information from previous human exposure to the NF or its source

XI. Nutritional information on the NF

XII. Microbiological information on the NF

XIII. Toxicological information on the NF

The information presented in this dossier is structured according to this scheme.

2. "Specifications of the NF"

2.1. Specifications

Specifications as submitted to JECFA for evaluation at its 55th meeting (June 6 – 15, 2000) are shown in Annex 2 of this document. Unless mentioned otherwise, the applied test methods are those specified in the JECFA Guide to Specifications (FAO Food and Nutrition Paper 5 Rev. 2).

Analyses of representative batches of trehalose demonstrate that trehalose produced at commercial scale complies with these specifications (Annex 3). Every batch of trehalose is tested for compliance with these specifications and is released for use in food only if the product meets all specified criteria.

In the pivotal safety studies (genotoxicity tests, embryotoxicity/teratogenicity studies in rats and rabbits, 2-generation reprotox study in rats, 3-month toxicity study in mice) trehalose was used which has been produced by the enzymatic process described in this dossier and complies with the specifications shown in Annex 2.

2.2. Physico-chemical properties

Solubility in water

(g anhydrous trehalose/100ml solution at 20°C): 43.0[3]

(g trehalose dihydrate/100ml solution at 20°C): 46.63

Specific rotation $[\alpha]_D^{20}$ (5% trehalose dihydrate, H_2O): +199°[4]

Melting point (°C): 210.5 (anhydrous)[5]; 97.0 (dihydrate)[6]

Heat of solution (kJ/mol): + 20.7 (trehalose dihydrate)

2.3. Other properties relevant to the use of trehalose as a food ingredient

2.3.1. Sweetness

Trehalose in aqueous solution (at concentrations from about 10-34% anhydrous trehalose) has a sweetness of about 40-45% relative to that of sucrose (Birch et al., 1970; Miyake, unpublished report). Correspondingly, the concentration at which a solution of trehalose is just perceived as sweet, is about two-times higher than that of sucrose (43.6 and 19 mM, respectively) (Lee & Birch, 1975). On the

[3] Lammert et al., 1998. A slightly lower solubility was reported by Sugimoto, 1995 and Miller et al., 1997.

[4] Values of +177° to +199° have been reported in the literature (Birch, 1963; Koch & Koch, 1925).

[5] Values of 203–216°C are given in the literature (Birch, 1965; Koch & Koch, 1925). More recently, the existence of three different polymorphs of anhydrous trehalose with different melting points has been reported, namely trehalose-β, trehalose-α, and trehalose-γ with m.p. 215, 125 and 120-130°C, respectively (Sussich et al., 1998).

[6] Values of 94-100°C have been reported in the literature (Birch, 1963).

other hand, the sweetness of trehalose has a longer persistence than that of sucrose (Portmann & Birch, 1995).

2.3.2. Hygroscopicity

Trehalose (dihydrate) has a very low hygroscopicity, similar to that of lactose (Miyake, unpublished report). Anhydrous (amorphous) trehalose absorbs moisture avidly until moisture content reaches a plateau at a concentration which is very similar to that of water in the dihydrate (10.5%) (Iglesias et al., 1997).

2.3.3. Chemical stability

Trehalose (dihydrate) is stable on storage at ambient temperature. In aqueous solution, trehalose is stable (i.e., is not hydrolyzed) in a pH range from 2 to 10 (4% solution, 100°C). As a non-reducing sugar, trehalose does not undergo Maillard reactions (Miyake, unpublished report).

2.3.4. Thermal stability

Under the temperature conditions applied typically during the processing and storage of food, trehalose is stable. As a non-reducing sugar, trehalose does not caramelize at elevated temperature.

3. "Effect of the production process applied to the NF"

3.1. Novelty of the process

Trehalose may be obtained by extraction from yeast. Trehalose obtained in this way was the subject of the evaluation by the ACNFP in 1990 (ACNFP 1990; 1991).

The subject of the present submission is a novel enzymatic process by which trehalose is produced from food-grade starch. Four enzymes are used in this process which have not been used hitherto in the EU in food or a food manufacturing process.

Trehalose produced by this enzymatic process is chemically identical to trehalose extracted from yeast as demonstrated by identical melting points and specific rotation[7].

[7] There exist three different isomers of trehalose: α,α-trehalose (trehalose) which is the subject of the present application; α,β-trehalose (neo-trehalose); and β,β-trehalose (isotrehalose). The three isomers have distinctly different specific rotations and melting points (Birch, 1963).

3.2. General description of the process

In a first step, starch is liquefied by treatment with a thermophilic α-amylase. In a second step, the obtained maltooligosaccharides are treated concurrently with maltooligosyl trehalose synthase (acronym: "MTSase")[8], maltooligosyl trehalose trehalohydrolase (acronym: "MTHase")[9], isoamylase, and cyclodextrin glucanotransferase (CGTase). The reactions catalyzed by MTSase and MTHase are depicted in Figure 2. Isoamylase is used as "debranching" enzyme, i.e., for cleaving α-1,6 glycosidic bonds of the starch molecule. CGTase is added in order to recycle maltose back into the process (Mandai et al., 1999). CGTase catalyzes intermolecular transglycosylation reactions in which maltose acts as an acceptor molecule (Tonkova, 1998; Brunet et al., 1998). Glucoamylase and α-amylase are added to release any remaining trehalose moieties and to degrade any remaining oligosaccharides and maltose to glucose. After completion of the trehalose forming enzymatic step (saccharification), the reaction mixture is decolorized with activated carbon, filtered using diatomaceous earth and perlite as filtering aid, de-ionized with ion exchange resins, and concentrated by evaporation. Trehalose is obtained by crystallization.

[8] Categorized as α-glucanotransferase in Japan. The systematic name is (1→4)-α-D-glucan 1-α-D-glucosylmutase (EC 5.4.99.15).

[9] Categorized as α-amylase in Japan. The systematic name is 4-α-D [(1→4)-α-D-glucano] trehalose glucanohydrolase (EC 3.2.1.141).

3.3. Detailed description of the process

The flow scheme of the trehalose production process is presented in Figure 3. The critical control points and control standards are shown in Annex 4.

3.3.1. Starch liquefaction

[This chapter reports on details of the production process for which the applicant claims confidentiality]

3.3.2. Saccharification, production of trehalose

[This chapter reports on details of the production process for which the applicant claims confidentiality]

3.3.3. Purification

[This chapter reports on details of the production process for which the applicant claims confidentiality]

3.3.4. Concentration and crystallization

[This chapter reports on details of the production process for which the applicant claims confidentiality]

3.4. Safety of raw material, chemicals, and enzymes used in the process

Food-grade starch is used as the starting material for the trehalose production process.

The chemicals used as processing aids in the manufacturing process (calcium carbonate, calcium chloride, hydrochloric acid, sodium hydroxide, sodium carbonate, activated carbon, perlite, diatomaceous earth) have a purity that makes them suitable for use in the present process (specifications are presented in Annex 4).

The applied cation and anion exchange resins comply with pertinent US regulations [21 CFR § 173.25 (ion exchange resins)] and the Council of Europe Resolution AP (97)1 (specifications are presented in Annex 4).

The thermophilic α-amylase (EC 3.2.1.1), which is used for starch liquefaction, is obtained from ***Bacillus licheniformis***. α-Amylase from this source microorganism has an "ADI not specified" (WHO, 1987). The enzyme applied in the present process complies with JECFA specifications (FAO, 1992b). In the US, a mixed carbohydrase and protease enzyme preparation from ***B. licheniformis*** has been affirmed as generally recognized as safe (GRAS) for use in the production of certain foods (21 CFR § 184.1027).

The MTSase (EC 5.4.99.15) and MTHase (EC 3.2.1.141), which are the crucial enzymes for the enzymatic synthesis of trehalose, are obtained from *Arthrobacter ramosus* (strain S34). The genus *Arthrobacter* is widely distributed in nature. Bacteria of this genus are predominant members of the soil microbiota (for taxonomic characteristics see Kvasnikov et al., 1986; Masiak et al., 1991). A few species of *Arthrobacter* have been identified in a small number of clinical samples but in no instance was *A. ramosus* isolated (Funke et al., 1996). *Arthrobacter* spp. is generally considered to be non-pathogenic and belongs consequently to risk group 1 of the German "Gentechnik-Sicherheitsverordnung" (GenTSV), i.e., Regulation on the Safety of Gentechnology. Group 1 comprises microorganisms which present no risk for humans and vertebrates. MTSase and MTHase are produced by cultivating *A. ramosus* (strain S34) in a standard medium containing food-grade maltose, food-grade mineral salts, a nitrogen source (peptone), and yeast extract. When the desired enzyme activity has been reached in the fermentation broth, albumen lysozyme is added in order to lyse the bacterial cells. The cells and particles are then removed by membrane filtration (pore size 0.04 μm). The filtrate is purified by cross-flow ultra-filtration (cut-off molecular weight of 10,000 daltons).

MTSase and MTHase have been purified and characterized. Their amino-acid composition has been determined (Nakada et al., 1995 a,b). The genetic sequence which encodes for the two enzymes has been identified as well (Maruta et al., 1996). Considering the generally recognized safety of *Arthrobacter* spp. and the efficacious

purification of trehalose which removes proteinaceous, glycidic, and ionic impurities almost quantitatively[10], the use of MTSase and MTHase as processing aids in the production of trehalose appears to be acceptable even in the absence of specific toxicological studies on these two enzymes (see Annex 3 for data demonstrating the absence of residual enzymatic activity in trehalose).

Isoamylase (EC 3.2.1.68) is used in the trehalose production process as debranching enzyme. The enzyme is obtained from a mutant strain of *Pseudomonas amyloderamosa*[11]. The extra-cellular enzyme is inducibly produced by the presence of maltose, maltodextrin or starch (Yokobayashi, 1988). For production of the enzyme at an appropriate scale, *P. amyloderamosa* is cultivated at about 30°C in a standard medium containing food-grade maltose, food-grade mineral salts, a nitrogen source (peptone), and yeast extract. When the desired enzyme activity has been reached, the fermentation broth is filtered (pore size 0.04 μm) and purified by cross-flow ultrafiltration (cut-off molecular weight of 10,000 daltons).

In anticipation of a direct food use of isoamylase which is unrelated to the subject of this application but which would require a full safety assessment of the enzyme, the safety of the isoamylase preparation was examined in standard Ames tests, an acute toxicity test in mice, and a 90-day oral toxicity study in rats. The Ames tests were conducted in *S. typhimurium* strains TA 1535, TA 1537, TA

[10] Analyses for protein of 5 different batches of trehalose using the Kjeldahl method gave values of 7-8 mg protein/100 g trehalose. For comparison, the starch used as a starting material has a protein content of ≤0.35%. The content of glycosidic impurities is described in chapter 3.5.2. The content of inorganic substances, as measured by the method for total ash, is not more than 50 mg/100 g trehalose (see chapter 3.5.1).

[11] *[This foot-note reports on details of the production process for which the applicant claims confidentiality]*

98, and TA 100, as well as in *E. coli* WP2 *uvrA* with and without metabolic activation. At levels of up to 5000 μg/plate, the test preparation was not toxic to the tester strains and was not mutagenic (van Delft, 1999).

The acute toxicity of *P. amyloderamosa* (wet cells), culture filtrate, and purified isoamylase was tested in mice. Groups of 10 male and 10 female mice received, by gavage, the wet cells at doses of 38.2 and 66.0 g/kg bw, the culture filtrate at doses of 34.7 and 60 ml/kg bw, and the isoamylase at doses of 12.0, 13.2, 14.5, 16.0, 17.6, 19.3 and 21.3 g/kg bw. The purified isoamylase was administered as a 40% suspension in water. No mortalities or clinical signs were observed after administration of the wet cells and the culture filtrate (Morimoto et al., 1979 a,b). In mice receiving the isoamylase suspension, mortalities occurred in a dose-dependent way starting from a dose of 14.5 g/kg bw mostly within the first 5 hours after gavage. The LD_{50} of purified isoamylase was about 17 g/kg bw. It is conceivable that the mortalities were the unspecific consequence of the forced ingestion of a thick suspension of protein (Morimoto et al., 1979 c).

In a subchronic (90-day) toxicity study 4 groups of rats (20/sex/group) received stabilized isoamylase preparation by gavage at levels of 0, 2.5, 5 and 10 ml/kg bw. The preparation had a protein content of 19 mg/ml. In order to administer the same volume of liquid (10 ml/kg bw) to all groups, the isoamylase preparation was diluted for the low and mid-dose group with control solution. The control group received 10 ml/kg bw of control solution. The general condition and behavior of the animals were not affected by the

treatment. Body weights did not differ between treatment groups and controls. Six rats died during the study. In 4 cases, the death resulted from a gavaging error (confirmed on autopsy). In the other two cases (one male rat of the mid and high dose group each) the cause of death could not be determined because of cannibalism and/or autolysis. Food intake, hematological parameters, clinico-chemical parameters, semi-quantitative urinalysis, organ weights at termination, and histopathological examination of organs and tissue did not reveal changes that could be attributed to the treatment. It was concluded the high-dose level tested (10 ml/kg bw) was a no-observed-adverse-effect level (NOAEL) (Lina, 1999).

On the basis of these results, the isoamylase preparation from *Pseudomonas amyloderamosa* appears to be fit for use in the trehalose production process.

CGTase (EC 2.4.1.19) is used in the trehalose production to increase the yield. The applied enzyme is obtained from a strain of *Bacillus stearothermophilus*. The safety of CGTase (from other source organisms) has been evaluated by JECFA in the context of the safety assessments of β- and γ-cyclodextrin (WHO, 1999). The safety of *B. stearothermophilus* as a source organism has been considered when the safety of α-amylase from this organism was assessed (WHO, 1991a). The non-pathogenic and non-toxicogenic properties of this microorganism were also appreciated in the evaluation of "maltogenic" amylase (WHO, 1998) and α-amylase (21 CFR 184.1012). It follows from these evaluations that CGTase from *B. stearothermophilus* can be safely used in the production of trehalose.

Glucoamylase (EC 3.2.1.3) from *Aspergillus niger* and α-amylase (EC 3.2.1.1) from *Bacillus subtilis* are used in the last step of the trehalose production process to degrade remaining oligosaccharides and maltose. The safety of these enzymes and source organisms has been evaluated by JECFA (WHO, 1972, 1988, 1991b) and tentative or final specifications have been laid down (FAO, 1992a, 1993). In the US, both enzymes are authorized for the treatment of wine (27 CFR § 24.246).

3.5. Potential impurities resulting from the production process

1.5.1. Impurities from raw material, process chemicals and enzymes

The food-grade starch contains small amounts of protein and inorganic salts. These natural impurities are removed almost completely during the different purification steps[10].

All applied chemicals are food-grade and/or have otherwise a high purity (see Annex 4). Residues of inorganic material would be detected in the tests for total ash. Lead would be detected in the specific test for lead. Specifications for arsenic are not included because there is no reason to expect the presence of this heavy metal in the product [JECFA, 53rd meeting, June 1999, Summary and Conclusions (item 5)].

The different enzyme preparations contain residues of the fermentation broth and by-products of the microbial metabolism. All ingredients of the fermentation broth are ordinary nutritional substances (carbohydrates, peptides, minerals). The bacterial fermentation of the carbohydrates which are added as a carbon source, yields typically short chain fatty acids. Toxic by-products are not known to be produced by the employed microorganisms. All substances carrying cationic or anionic functional groups are expected to be removed by the de-ionization step using ion-exchange resins. An additional purification is obtained by the treatment with activated carbon. Any remaining organic impurities are likely to be removed during the crystallization of trehalose. Inorganic residues would be detected by the test for total ash.

3.5.2. By-products from enzymatic saccharification

The treatment of the enzymatic depolymerisation products of starch (maltooligosaccharides) with MTSase, MTHase, isoamylase and CGTase results in the formation of trehalose, and smaller amounts of glucose, maltose and some tri- and higher maltooligosaccharides. The final treatment of this reaction mixture with glucoamylase and α-amylase removes maltose and the oligosaccharides almost completely. The resulting glucose and any remaining maltose and oligosaccharides are removed during the crystallization of trehalose. On HPLC analysis of crystalline trehalose produced according to this process, only three glycosidic impurities could be detected, namely glucose, glucosyltrehalose (syn: α-D-maltosyl-α-D-glucoside)[12], and

[12] The physicochemical properties of glucosyltrehalose have been determined. The LD_{50} of this trisaccharide is ≥ 50 g/kg bw in mice (Tabuchi et al., 1999). An enzyme preparation from rat small intestine hydrolyzed the substance to glucose and trehalose (Kurimoto et al., 1997b; Mandai et al., 1999).

α-D-isomaltosyl-α-D-glucoside[13]. In a trehalose batch with a purity of 99.1% (as determined by HPLC) these three by-products occurred at levels of 0.5, 0.3 and 0.1%, respectively. Other mono-, di- and oligosaccharides were not detected.

3.5.3. Purification steps

The proteinaceous impurities from the raw-material (starch) and the different enzyme preparations are removed by heat denaturation followed by treatment with activated carbon and filtration[10,14]. The treatment with activated carbon will also remove the main part of other organic, non-ionic impurities.

The ionic impurities are removed in the two-step demineralization procedure using cation and anion exchange resins. Any remaining inorganic salts would be detected by the test for total ash. Lead would be detected by the specific test for lead.

The glycosidic impurities and extractives of the ion-exchange resins are removed during the crystallization of trehalose. Excessive amounts of glycosidic impurities would be detected in the HPLC analysis which forms the basis for the Assay.

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[13] α-Isomaltosyl-α-D-glucoside is digested by an enzyme preparation from rat small intestine. Non-digested product is fermented by intestinal bacteria (e.g., ***Bifidobacteria***) (Kurimoto et al., 1997a).

[14] Five batches of trehalose were analyzed for residual enzymatic activity of α-amylase, CGTase, MTSase, isoamylase, and glucoamylase using ELISA assays (Nagaoka et al., 1997). (The enzymes used for the immunization were the ones used in trehalose production and had an identical purity). No enzymatic activity was detected (limit of detection 1 mU/g trehalose for all enzymes except MTSase (10 mU/g trehalose) (Annex 4).

It is unlikely that microorganisms could be carried into the process (mainly from the raw material) and survive the production process which involves several heat treatment steps. Any surviving microorganisms would be detected in the tests for total (aerobic) plate counts, *E. coli*, *Salmonella*, and yeast and molds.

4. "History of the organism used as the source of the NF"

In the novel production process which is the subject of this application, trehalose is produced enzymatically from food-grade starch, i.e., it is not obtained from a plant, animal, or microorganism. Consequently, no information is required under this heading.

5. "Anticipated intake / extent of use of the NF"

5.1. Intended uses in food

Being about 40-45% as sweet as sucrose (Birch et al., 1970; Sugimoto, 1995), trehalose may be used to replace some of the sucrose in those types of food which require a certain amount of sucrose for technological reasons but would have a more balanced taste profile if their sweetness was somewhat reduced.

Trehalose can be used to make fruit fillings and toppings, cream fillings, etc. which are microbiologically and physically as stable as those produced with sucrose but which have a richer flavor because trehalose has a lower sweetness. In fruit preparations with a naturally high acid content there is less browning with trehalose than with sucrose because trehalose is more resistant to acid-catalyzed hydrolysis and does not participate in Maillard-type reactions (O'Brien, 1996; Miyake, unpublished report).

Trehalose acts as a stabilizer for proteins during freezing and drying. It has, for example, been found that enzymes retained a higher activity if they were dried in the presence of trehalose (Colaço et al., 1992, 1994). Trehalose also stabilizes phospholipid bilayers (e.g., liposomes) and more complex biological structures (Crowe et al., 1988; Leslie et al, 1995; Aguilera & Karel, 1997). In food processing, trehalose has, therefore, been used in the preparation of egg powder and dried fruits (Roser, 1991; Colaço & Roser, 1995; Miyake, unpublished report). As a cryoprotecant it is used in the production of surimi, other restructured sea food, frozen gelatin des-

serts, and premium ice creams. Trehalose appears to have a stabilizing effect not only during dehydration but also during the rehydration of susceptible molecules. Incorporated in instant noodles or precooked rice, it accelerates the rehydration of the product.

Sugars may inhibit the retrogradation of starch in bread, cake, or other starch-based foods. In other words, sugars may stabilize the quality and prevent staleness of such foods. In a model system with gelatinized starch as well as in sponge cake and baked rolls, trehalose was found to be more efficient in this respect than sucrose (Miyake, unpublished report). In cookies, a portion of the fat can be replaced by trehalose without compromising the texture and shelf-life of the product.

Trehalose possesses a significantly higher glass transition temperature than maltose, sucrose or glucose (Green & Angell, 1989; Mehl, 1997). It is, therefore, well suited for the production of transparent, hard-boiled candies and for use in coatings.

Because of its high chemical stability and low hygroscopicity, trehalose is well-suited for the production of compressed tablets. In some cases, the stabilizing effect of trehalose on sensitive ingredients of such tablets offers an additional benefit.

The low-hygroscopicity of trehalose makes it potential substitute for other sugars which are presently used for icings, dusting, and coatings.

5.2. Current food applications of trehalose in Japan

In Japan, trehalose is included under No 303 in the "List of Existing Food Additives". This list comprises naturally occurring additives which may not be produced, however, by chemical processes. The listed products may be used in foods generally, unless restrictions are specified (List of Existing Food Additives, published by the Ministry of Health and Welfare, April 16, 1996).

Trehalose is used mainly in bakery products (cakes, frozen bread dough, cream fillings, toppings, etc.), beverages (sports drinks, fruit drinks), hard and soft confectionery, fruit jam, breakfast cereals, rice, and noodles. The main purposes of use are the reduction of sweetness (in bakery products and confectionery), the reduction of moisture absorption (in breakfast cereals and certain types of confectionery), the reduction of browning reactions (in beverages and certain types of confectionery) and the prevention of starch retrogradation (in bakery products and noodles). A list of typical applications and use levels in Japanese foods is presented in Annex 5.

5.3. Estimated daily intake

The estimated daily intake (EDI) of trehalose from its different projected uses in food (as specified in Table 1) but excluding chewing gum, has been calculated for the US population by ENVIRON (Arlington, VA) using the dietary survey approach (Annex 6). Although dietary habits of US and European consumers differ somewhat,

an EDI calculation on basis of US data was considered adequate because the consumption of processed food is rather higher in the US than in the EU and because European food intake data are not available for conducting EDI calculations of a similar degree of refinement. This calculation model relies on food consumption data from the 1994-96 Continuing Survey of Food Intakes by Individuals (94-96 CSFII) in which data were collected from a representative sample of individuals residing in households in the US (approximately 15,000 individuals). Each individual was surveyed for two non-consecutive days using 24-hour recall interviews. The foods consumed were coded according to a system which contains about 6,000 different categories.

For the purpose of the present EDI calculation it was assumed that each food (or food component) which may contain trehalose, indeed contained trehalose at the highest, feasible concentration (as specified in Table 1). Where trehalose was used in a component of the food (e.g., in fruit-fillings), the intake of that component was calculated from data on food composition.

The EDI of trehalose was calculated for each food category in which trehalose may be used, and for all these food categories combined (except for chewing gum for which such data are not available). Mean and 90th percentile intakes were calculated on per-user basis for the following age groups: children 2-12 years of age; teenagers 13-19 years of age; and the adult U.S. population 20+ years of age. "Users" were defined as individuals who consumed food in the particular category on at least one occasion. Since food intake was recorded by time of day and by eating occasion [breakfast, brunch, lunch, dinner, supper, snack, and other (extended) eating occa-

sion], the intake of trehalose could be calculated per eating occasion.

The main results of these calculations are presented in Table 2. Details are shown in Annex 6. For adults, the estimated exposure to trehalose from all proposed uses, excluding chewing gum, is 5.6 and 13.0 g/day at the mean and 90th percentile, respectively. Mean intake by eating occasion (excluding extended eating occasions) ranged from 3.9 to 8.1 g/occasion, while intake at the 90th percentile ranged from 7.6 to 18.6 g/occasion. The highest estimated exposure to trehalose results from the intake of ice cream. In teenagers, this product results in an average intake of 16.7 g/day (intake may occur at more than one eating occasion) (see Annex 6).

Trehalose may be used as a non-hygroscopic sweetener in the coating of chewing gum. In this application, trehalose would account for not more than 10% of the total weight of the chewing gum. The estimation of trehalose intake from chewing gum is based on a separate survey in which approximately 1,500 individuals reported their one-day intake of regular and sugarfree gum by mail. The survey, which was conducted in 1995, distinguished between 2 age groups (children and adults) but the amount of coated chewing gum consumption did not differ between these two groups. From the results of this survey it is concluded that the average user of coated chewing gum would ingest 0.4 g/d trehalose from this product (90th percentile consumer: 0.8 g/d) (Annex 6).

In assessing the total daily intake of trehalose from all dietary sources it is important to note that, with regard to gastrointestinal tolerance, the intake per eating occasion is a more important

parameter than the total daily intake from all dietary sources combined. Considering the different anticipated uses of trehalose (as described in chapter 5.1), it becomes apparent that the intake of trehalose is not concentrated to certain eating occasions (such as main meals) but is spread evenly over the day by the mere nature of the products in which it is used. This is also reflected by the data on estimated daily intakes. The figures shown in Table 2 demonstrate that the mean and 90th percentile intake per eating occasion do not exceed 8.1 and 18.6 g, respectively, in any age group. A comparison between the intakes from the various food categories and the total intake from all sources demonstrates that many uses are mutually exclusive. In other words, a consumer of a sponge cake is unlikely to eat a fruit pie at the same eating occasion. Consequently, trehalose intakes per eating occasion are similar to the intake from one specific food category in a given age group (Annex 6).

Estimated intakes per eating occasion are far below the 50-g intake which is typically used in trehalose loading tests and which is generally well tolerated (Bolte et al., 1973). The intakes per eating occasion are also below the threshold dose for abdominal effects in particularly sensitive subjects [≥30 g per eating occasion (Oku & Okazaki, 1998)]. Adverse gastrointestinal side-effects from the intended uses of trehalose are, therefore, not to be expected. Since in some applications trehalose may substitute for polyols, the total intake of low-digestible carbohydrates could even slightly decrease.

6. "Information from previous human exposure to the NF or its source"

6.1. Occurrence in nature

Trehalose occurs widespread in nature. Except for a few rare cases, only the α,α-isomer has been found (Elbein, 1974). Trehalose occurs as such in the cells of a variety of plants and in the blood (hemolymph) of arthropods. In *Mycobacteriae* (e.g., *M. tuberculosis*), it occurs in the form of a glycolipid, namely the 6,6'-di-ester of mycolic acid (for references see Birch, 1963). Esters of other fatty acids with trehalose have been identified in other bacteria (for references see Birch, 1963).

6.1.1. Occurrence in plants

Trehalose occurs in bacteria[15], yeast (e.g., *S. cerevisiae*)[16], a wide variety of fungi[17], algae[18], and a few higher plants[19] (reviewed by Elbein, 1974).

Intracellular trehalose appears to play an important role in the protection of the cells from dehydration and freezing, as well as

[15] Nicolaus et al., 1988; Stjernholm, 1958 cited in Birch, 1963; Streeter & Bhagwat, 1999.

[16] Koch & Koch, 1925; Oku et al., 1998.

[17] Elbein, 1974; Thevelein, 1984; Oku et al., 1998.

[18] Myrbäck, 1949; Elander & Myrbäck, 1949 cited in Birch, 1963; Lindberg, 1955 cited in Birch, 1963.

[19] Oesch & Meier, 1967 cited in Elbein, 1974; Goddijn & Smeekens, 1998; Oku et al., 1998; Ghasempour et al., 1998; Müller et al., 1999.

from other adverse environmental conditions (e.g., heat shock, toxic levels of ethanol, osmostress) (Singer & Lindquist, 1998a, b). In addition, trehalose may serve as reserve carbohydrate during periods of carbon starvation (Silljé et al., 1999).

Because of its presence in baker's and brewer's yeast, in which it reaches concentrations of up to 23% on a dry weight basis[20], small amounts of trehalose have been found in bread (1.2-1.5 g/kg dry substance)[21], beer (45-240 mg/l)[21], wine (44-129 mg/l)[21], and honey (0.1-2.3 g/100g)[22].

Mushrooms, including many edible species, contain trehalose at levels of about 2-12 g/100g dry weight, but contents of up to 22% have also been reported (Oku et al., 1998; for additional references see Elbein, 1974).

6.1.2. Occurrence in animals

Trehalose has been found in many species of insects (Elbein, 1974; Becker et al., 1996). It has also been detected in a variety of other invertebrates, including nematodes[23], crustaceans, and annelids (reviewed by Elbein, 1974). The blood of lobsters (*Homarus*

[20] Myrbäck & Örtenblad, 1936 cited in Birch, 1963; Steiner & Cori, 1935; Stewart et al., 1950.

[21] Oku et al., 1998.

[22] Mendes et al., 1998.

[23] Behm, 1997.

americanus) and crabs (*Carcinus macenus*) contains trehalose at concentrations of 2.5 and 1.5 mg/100ml, respectively.

In many insects, trehalose is the major sugar found in the blood (hemolymph) at concentrations of between 1 to 2% (Wyatt & Kalf, 1957; Thompson, 1999). It occurs at all stages of the development (pupal, larval, and adult stage) but concentrations may differ. In flying insects, trehalose functions as the energy source to sustain flight (Becker et al., 1996; Candy et al., 1997). Trehalose may also play a role in the frost resistance of some insects (overwintering prepupal larvae of the sawfly contain 5-9% trehalose) and nematodes [juveniles of an entomopathogenic nematode increase their trehalose levels with decreasing temperature [6% trehalose (% dry weight) at 5-8°C] (Asahino & Tanno, 1964 cited in Elbein, 1974; Qiu & Bedding, 1999).

High concentrations of trehalose (7%) were found in a type of manna which was used by Bedouins as a sweetener for coffee in the North Iraqian desert (Leibowitz, 1943, 1944). The manna is probably the excretion product of some scale insects.

6.2. Consumption of trehalose added to food in Japan

Trehalose produced by the enzymatic process described in this dossier became available in Japan for food use in 1995. By mid 1997 monthly sales reached about 500 tons.

6.3. Intake of high single doses in trehalose tolerance tests

The absorptive capacity of the small intestine may be examined in patients suffering from malabsorption syndrom by measuring the absorption of ingested xylose or trehalose (Dominick & Anspach, 1975). Typically, rather high doses are administered (e.g., 50 g/person or 1 g/kg bw). The results that were obtained from such tests with trehalose are described in chapters 9.2.1 and 9.4.1.

7. "Nutritional information on the NF"

Ingested trehalose is digested by trehalase in the small intestine to glucose which is readily absorbed (see chapter 9.2.1). Trehalose has, therefore, the same physiological energy value as glucose or maltose. The metabolic pathways of trehalose and maltose (or starch after digestion by amylase) merge after digestion to glucose at the brush border of the intestinal mucosa.

It follows that trehalose is nutritionally equivalent to glucose and maltose, as well as maltodextrin and starch. (Maltose is the main product of the digestion of the latter two products by pancreatic amylase).

Trehalose may substitute for some of the sucrose in certain foods (see chapter 5.1 for examples). In comparison to sucrose, trehalose provides on digestion only glucose but no fructose. In terms of physiological energy (caloric value), this does not make a difference. In terms of nutritional and metabolic properties, no disadvantage is associated with a reduced intake of fructose.

8. "Microbiological information on the NF"

The enzymes which are used in the novel production process of trehalose, are obtained from non-genetically modified strains of *Arthrobacter ramosus, Pseudomonas amyloderamosa, Bacillus stearothermophilus, Aspergillus niger, and Bacillus subtilis*.

The enzyme-containing fermentation broths are separated from the source microorganisms by filtration. Moreover, the trehalose production process comprises several steps with heat-treatment. Therefore, it is unlikely that any of the source microorganisms would be present in the final product. An inadvertent presence of microorganisms would be detected by the applied quality control procedures. The trehalose solutions obtained from the de-ionization step and the subsequent concentration step are controlled regularly in process for microbiological contamination. The specifications of the end-product also include microbiological parameters.

The safety of the applied enzymes and their source microorganisms has been discussed in chapter 3.4.

9. "Toxicological information on the NF"

9.1. General remarks

Ingested trehalose enters the animal or human body after digestion in the form of glucose. From a metabolic and toxicological perspective, glucose, maltose, maltodextrin and starch represent, therefore, counterparts which may be used as baseline to facilitate the safety assessment.

The novel process by which trehalose is produced and which is the subject of the present application, does not introduce by-products that are potentially toxic as explained in chapters 3.4 and 3.5.

Allergic reactions to the intake of trehalose produced by the enzymatic process are not to be expected. Sugars generally have a very low allergenic potential. The by-products which are present in trehalose in very small concentrations, are glucose, maltose and maltooligosaccharides which are not known to be allergenic. Residual activity of the enzymes used in the production process was not detected (see chapters 3.5.2 and 3.5.3). Proteinaceous impurities which are present in trehalose at very low concentrations (70 – 80 ppm, see chapter 3.4) stem most likely from the applied raw material, i.e. food grade starch, which has a protein content of $\leq$ 3500 ppm).

9.2. Metabolic studies on trehalose

In humans and many animals, the fate of ingested or parenterally administered trehalose corresponds to that of glucose because trehalose is rapidly hydrolyzed to glucose by trehalase (EC 3.2.1.28). Trehalase occurs in humans and most animals at the brush-border of the intestinal mucosa and, depending upon the species, also in the proximal tubular cells of the kidney, as well as in the liver and the blood plasma (Courtois & Demelier, 1966; Hore & Messer, 1968; Berg et al., 1973; Demelier et al., 1975; Vázquez et al., 1975; Labat-Robert, 1982; van Handel, 1970; Rodeck & Dominick, 1983; Niederst & Dauça, 1985; Jönsson et al., 1986; Eze, 1989; Riby et al., 1990; Yoshida, 1993; Isichei & Gorecki, 1993).

9.2.1. Digestion and absorption of ingested trehalose

Intestinal trehalase is a membrane-bound enzyme. It is localized in the brush border of the mucosal cells of the duodenum, jejunum, and – at lower levels – the ileum (Hietanen, 1973). Trehalase accounts for all the trehalase activity of the small intestinal mucosa (i.e., there are no other glycosidases of mammalian origin which could hydrolyze trehalose). Trehalase activity was found in the small intestine of many animals (e.g., mouse, rat, guinea pig, rabbit, pig, baboon) and humans (Galand, 1989; Maestracci, 1976; Ruppin et al., 1974; Hietanen, 1973; Cerda et al., 1972). Only a few species appear to lack intestinal trehalase (e.g., cat) (Hore & Messer, 1968; Galand, 1989; Hietanen, 1973).

A very small fraction of the healthy human population exhibits a primary (hereditary) or secondary (acquired) trehalase deficiency (see chapter 9.4.2). People with this condition may experience intestinal side-effects (flatulence, laxation) after the ingestion of excessive amounts of trehalose, like lactase-deficient people after the intake of excessive amounts of lactose. In this regard it is noteworthy, however, that the incidence of lactase deficiency is higher than that of trehalase deficiency (Gudmand-Høyer & Skovbjerg, 1996).

If, under conditions of a relative or absolute deficiency of intestinal trehalase, ingested trehalose is incompletely digested to glucose at the small intestinal mucosa, a small fraction (about 0.5%) may be absorbed by passive diffusion as shown for other disaccharides (e.g., lactulose) (van Elburg et al., 1995)[24]. Any trehalose not digested but absorbed would then be split to glucose by trehalase in the liver, plasma and/or kidney, or would be excreted unchanged with the urine (Demelier et al., 1975). The not digested and not absorbed portion of ingested trehalose will likely be fermented by the intestinal microflora to short-chain fatty acids (mainly acetate, propionate, butyrate) as has been shown for many other non-digestible carbohydrates (e.g., resistant starch, inulin, etc.).

The following experimental data demonstrate that ingested trehalose is efficiently digested to glucose which is then absorbed and metabolized in animals and humans.

[24] Since trehalose appears to have a higher hydrated volume than other sugars, the fractional absorption by passive diffusion may actually be lower for trehalose than for other disaccharides (Sola-Penna & Meyer-Fernandes, 1998).

In an early study on the metabolism of different rare sugars, groups of fasted rats received about 0.5 g glucose, trehalose, and other sugars by gavage. An additional group served as control. The animals were killed 8-9 hours after dosing and liver glycogen was determined. In comparison to the untreated controls, the average liver glycogen content was increased after trehalose ingestion suggesting that trehalose was efficiently converted to glucose (Clarke et al., 1939).

In the context of an acute toxicity test, four male Beagle dogs received trehalose *per os* (5 g/kg bw). Blood and urine were collected before and in regular intervals after dosing for analysis of serum glucose and trehalose. Serum glucose increased markedly reaching maximum levels after 1 hour. Urinary glucose levels increased transiently above baseline levels (presumably due to the hydrolysis of some absorbed trehalose in the kidney). Urinary trehalose was not detected (Atkinson & Thomas, 1994c)[25].

Fifty healthy subjects ingested solutions with 50 g trehalose and 50 g glucose on different days after an overnight fast. Blood was collected before and 15, 30, 60, 90, and 120 min after dosing. The fractional intestinal digestion of trehalose to absorbed glucose was calculated from the ratio of the area under the blood glucose curve (0-60 min) of trehalose relative to that of glucose. The observed values ranged from 0.3 – 1.5 with a median of 0.7 (Bergoz, 1971, Bergoz et al., 1973).

[25] Similar measurements were made in the context of acute toxicity studies in mice and rats. However, the number of urine samples available for analysis was too small to obtain meaningful results (Atkinson & Thomas, 1994a, b).

Using an identical experimental protocol, the trehalose absorption was studied in 9 uremic patients suffering from chronic renal insufficiency. The absorption of trehalose relative to that of glucose was 0.83 $\pm$ 0.5 (Pointner et al., 1974).

The digestion of trehalose in children is similarly high. Sixteen healthy children (age 1 – 12 years) ingested trehalose and glucose at a dose of 1 g/kg bw. The ratio of the area under the curve of blood glucose (0 – 60 min) was 0.91 (mean). Values below 0.6 were found only in children suffering from different forms of intestinal disease (Dominick & Anspach, 1975).

Sixty healthy fasted volunteers received on different days 50 g trehalose, glucose, lactose and sucrose (dissolved in 400 ml water). Blood was collected for determination of glucose before and 30, 60, 90 and 120 min after dosing. Symptoms of gastrointestinal intolerance were not reported after intake of trehalose. An evaluation of the blood glucose profiles indicated that none of the volunteers suffered from trehalose malabsorption [ratio of AUC (trehalose)/AUC (glucose) > 0.3] (Bolte et al., 1973). The reported digestion and absorption rate (median 0.7) is similar to that reported by Bergoz (1971, 1973) but is slightly lower than that observed. In this regard, it must be noted, however, that the ingestion of 400 ml water with 50 g trehalose on an empty stomach represents an extreme situation which is highly unlikely to occur under ordinary conditions of use of trehalose as a food ingredient (see chapter 5.3). Typically, trehalose will be ingested at lower doses and in most instances together with other foods. Under these conditions, the small intestinal transit-time will be longer and hence digestion of trehalose to absorbable glucose will be more complete (Heine et al., 1996).

9.2.2. Metabolism of parenterally administered trehalose

Trehalose which enters the circulating blood either through passive absorption from the gut or by systemic administration, will be converted to glucose by trehalase which, depending upon species, occurs in the serum, kidney liver, and bile (Van Handel, 1969; Labat-Robert, 1982; Arola et al., 1999). Any trehalose that would escape hydrolysis by plasma and liver trehalase would be excreted in the primary urine. In mice, rabbits, dogs, pigs and humans, excreted trehalose would be cleaved by renal trehalase which is located in the brush border of the proximal tubular cells (Demelier et al., 1975; Niederst & Dauça, 1985; Riby et al., 1990)[26]. In rats, guinea pigs, birds, and a few other species which appear to lack renal trehalase (van Handel, 1969; Demelier et al., 1975; Riby et al., 1990), ultrafiltered trehalose would be excreted with the urine.

The following experimental data illustrate the efficacy of trehalose elimination from the circulating blood.

Rats, guinea pigs and rabbits were given trehalose at a dose of 0.5 or 1 g/kg bw by intravenous injection. In rats, about 87% (range 75-98%) of the administered dose was recovered from the urine indicating that trehalase activity in the liver and kidneys was low or absent. In the guinea pigs and rabbits, only 7-9% of the dose were ex-

[26] Under physiological conditions, small amounts of trehalase are excreted with the human urine (Maruhn et al., 1976). Increased levels of urinary trehalase are indicative of proximal tubular damage (Sasai-Takedatsu et al., 1996).

creted with the urine. Examination of the trehalose levels drawn from the renal artery and vein of rabbits indicated that trehalose was hydrolyzed efficiently in the kidneys (Demelier et al., 1975).

Rabbits given intravenous injections of 500 mg trehalose (equivalent to 200-300 mg/kg) were able to clear the sugar from their plasma within 60 minutes. No trehalose was found in the urine. Only a small amount of trehalose was detected in the urine of rabbits receiving an intravenous dose of 1,000 mg. By contrast, rats given 100 mg (300 mg/kg) removed the sugar from the plasma at approximately the same rate as the rabbits, but trehalose was recovered from the urine in proportion to the plasma concentrations. These results indicated that there was no renal metabolism of trehalose in the rat (Riby et al., 1990).

Male rabbits received infusions of trehalose solution (10%), maltose solution (10%), glucose solution (5%), or saline for 90 min at a rate of 6.7 ml/kg bw/h (4 rabbits per treatment). Blood was collected before and 30, 60, 90, 120 and 180 min after start of the infusion. Urine was collected over 24 hours from start of the infusion. Serum glucose, trehalose, maltose, and insulin were determined. The urine was analyzed for glucose, trehalose, and maltose. The infusion of trehalose led to a rapid increase of serum glucose levels which peaked at 90 min (end of infusion) and returned back to baseline values within 90 min after end of the infusion. Serum insulin levels exhibited a corresponding time course. The urinary glucose excretion was slightly higher in trehalose infused rabbits than in glucose or saline infused rabbits (difference not significant with the low number of animals used). About 1% of the infused trehalose was recovered in the urine. The data on serum and urinary sugars, and on serum insulin demonstrated that infused trehalose is me-

tabolized more rapidly and more completely than infused maltose at the applied infusion rate. The better utilization of trehalose was confirmed in an experiment with infusion of trehalose and maltose to alloxan-diabetic rabbits. In a further experiment, the continuous infusion of trehalose or glucose (supplemented with amino acids) for 5 days at a rate of 8.23 g/kg bw/d was not associated with any signs of toxicity or adverse effects on serum biochemical parameters of male rabbits (Sato et al., 1999).

In the context of an acute toxicity test, four male Beagle dogs received trehalose by intravenous injection (1 g/kg bw). Blood and urine were collected before and in regular intervals after dosing for analysis of serum glucose and trehalose. Serum glucose raised slightly reaching highest values after about 30-60 minutes of dosing. Urinary glucose was increased markedly probably reflecting hydrolysis of trehalose by renal trehalose in the proximal tubuli. The trehalose concentration in the urine 5 min after dosing was markedly higher than that found in the plasma which suggests that trehalose is rapidly cleared by the kidneys (Atkinson & Thomas, 1994c).

9.3. Toxicological studies

9.3.1. Acute toxicity

The acute toxicity of trehalose was examined in mice, rats, and dogs. The results are summarized in Table 3.

In the rodent studies conducted by the Frederick Research Center, groups of 5 animals of each sex were tested at one dose level (i.v., 1 g/kg bw; p.o., 5 g/kg bw) or served as controls. In the dog study conducted by the same contract institute, 4 male Beagles received a single i.v. dose of 1 g/kg bw, followed 6 days later by an oral dose of 5 g/kg bw. There were no mortalities due to the trehalose treatments in any species. Signs of systemic toxicity were not observed. Diarrhoea did not occur in response to the oral administration of trehalose. Body weight gains were not impaired by the treatment during the 7-day (dogs) or 14-day (rodents) post-treatment period (Atkinson & Thomas, 1994 a, b, c).

In a subsequent acute toxicity test in rats, trehalose was administered by gavage at a dose of 16 g/kg bw to five fasted Sprague-Dawley rats of each sex. The dose was given in two equal portions with a 1-hour period between administrations. No deaths occurred in response to the treatment. Soft to liquid feces occurred in one male on day 1 of the study. Piloerection was observed in all rats during day 1. Body weight gains were within normal limits during the 14-day post-treatment period. Gross necropsy on day 15 revealed no abnormalities (Mc Rae, 1995).

9.3.2. Short-term studies of toxicity

Mice

Groups of ten CD-1 mice of each sex were given trehalose at doses of 1 g/kg bw/d by intravenous injection, 5 g/kg bw/d by gavage, or 2.5 g/kg bw/d by subcutaneous injection for 14 consecutive days. An additional group (control group) received sterile saline (same dose volume as the oral treatment group). Body weights, weight gains, and

food consumption did not differ between the treatment groups over the 2-week treatment period. One female of the i.v. group died after dosing on day 6 (necropsy findings were unremarkable). No clinical signs of toxicity were seen throughout the study. Mild necrosis and/or local irritation of the tail in some animals of the i.v. group was ascribed to the repeated administration of a hypertonic solution. Blood samples were collected at termination for analysis of standard hematological and biochemical parameters (5 animals/sex for each set of parameters). The white blood cell counts were below control levels in males but not females of the p.o. and s.c. groups. Differences between treated groups and controls of some biochemical parameters were quantitatively small and also limited to one sex (males). Macroscopic and histopathological examination of all tissues of p.o. treated mice did not reveal any changes that could be attributed to the exposure to trehalose (Atkinson & Thomas, 1994d).

Four groups of HanIbm:NMRI mice (20 mice/sex/group) received diets with admixture of 0 (control), 0.5, 1.5 and 5% trehalose for 13 weeks. Animals were examined for clinical signs of toxicity; body weights and food consumption were monitored throughout the study. Ophthalmoscopic examinations were performed on 10 mice/sex of the control and high-dose group before start and in the final week of the study. Blood samples were collected for analysis of standard hematological and biochemical parameters from fasted animals (10 mice/sex/group) in weeks 5, 9, and 13 (termination). Urine was collected during the 18-hour fasting period for measurement of pH, standard semi-quantitative urinary parameters, and microscopic examination of the sediment. After at least 90 days of treatment, the animals were killed and subjected to gross necropsy. The absolute and relative weights of main organs were determined. Tissues and organs of the control and high dose group (and those exhibiting macro-

scopic abnormalities of the low and mid-dose group) were examined microscopically for pathological changes.

There were four deaths during the study (1 spontaneous death in the control group, 1 male of the high-dose group killed *in extremis* in week 12, and two deaths in connection with blood sampling). The animal of the high-dose group exhibited severe pyelonephritis on histopathologic examination which was considered not to be related to the treatment. Food consumption and body weight gains were slightly below controls in males of the mid- and high-dose groups. Treatment related clinical signs were not observed. Ophthalmoscopic examination revealed no treatment-related effects. Hematological parameters remained unaffected by the treatment. Plasma glucose levels tended to be slightly increased in males and females of the high dose group (significant in females in week 5 and 9). In the females, plasma phosphate was increased at the same time. In week 13, plasma potassium was decreased in a dose-related manner in males and females, reaching statistical significance in the high-dose group. However, the observed values were still well within the limits of historical controls. Significant changes of a few other parameters were considered coincidental since they occurred only at one occasion and/or in one sex. Urinary parameters did not reveal changes that could be attributed to the treatment. Organ weights were unaffected by the treatment. There were no macroscopic or microscopic findings in organs and tissues which were considered to be related to the treatment. The high-dose level [equivalent to 7.3 (males) and 9.3 (females) g/kg bw/d] was considered to be tolerated without toxicological effects (Schmid et al., 1998).

Dogs

Groups of three male and three female Beagle dogs received trehalose at doses of 1 g/kg bw/d by intravenous injection in the cephalic vein, 5 g/kg/d by oral capsule, or 0.25 g/kg/d by subcutaneous injection for 14 consecutive days. A control group was given empty capsules at the same number of capsules/kg body weight as those treated with the test article. All animals survived till the end of the study. Body weights and food consumption did not differ between controls and the different treatment groups. All dogs of the p.o. group experienced diarrhoea on some days of the study (range: 4-8 days out of 14 days). Vomiting was observed in a few animals of the control, p.o. and s.c. groups. Measurement of serum glucose after dosing at the last treatment day revealed significant transient increases in i.v. and p.o. dosed dogs. Analysis of blood samples collected at termination did not reveal toxicologically relevant changes of hematological or biochemical parameters. Macroscopic examination at necropsy and microscopic examination of standard organs and tissue did not reveal changes which could be attributed to the treatment. It was concluded that trehalose was not toxic at the applied doses (Atkinson & Thomas, 1994e).

9.3.3. Genotoxicity

The results of assays for genotoxicity are shown in Table 4.

9.3.4. Developmental toxicity

Rats

In a study of embryotoxicity and teratogenicity, groups of 28 presumed pregnant Wistar Crl:(WI)WU BR rats were fed diets containing trehalose (purity: 99%) at concentrations of 0, 2.5, 5 and 10% on days 0-21 of gestation. Trehalose was added to the test diets in lieu of pre-gelatinized starch. The animals were examined throughout the study, and body weight and food consumption were recorded. The rats were killed on day 21 and examined for parameters of reproductive performance. Fetuses were examined for signs of toxicity, external malformations, and soft-tissue defects and were stained for detection of skeletal anomalies.

No deaths occurred during the study. Food consumption and maternal body-weight gain were similar in all groups. The trehalose intake during the gestation period ranged from 1.4-2.0, 2.8-3.9 and 5.5-7.8 g trehalose/kg bw/d for the low, mid and high-dose group, respectively. Early delivery was observed in 1 female of the control group and 2 females of the low-dose group. Necropsy of the dams showed no adverse effects that could be related to the treatment. The number of viable litters, the number of corpora lutea, and the mean number of implantation sites were similar in all groups. Fetal length and body weight were also similar in all groups. Examination of the fetuses of the control and high-dose group revealed no treatment-related increase in gross, skeletal, or visceral abnormalities. Under the conditions of this assay, trehalose was not teratogenic and did not induce any maternal or developmental toxicity (Waalkens-Berendsen, 1998).

In a 2-geneneration reproductive toxicity study, groups of Wistar rats (Crl:(WI)WU BR) (28 rats/sex/group) were administered a diet containing trehalose (purity 99%) at concentrations of 0, 2.5, 5 or 10%. After a period of 10 weeks (pre-mating period), each female was caged with one male of the same treatment group until pregnancy occurred. Upon evidence of copulation, the females were housed individually. Trehalose treatment was continued during pregnancy and lactation. On day 4 after delivery, the litters were culled to 8 pups. After weaning, 28 males and 28 females were selected at random from as many litters as possible in each group and raised to maturity while continuing the trehalose treatment. These animals (F_1 generation) were then used to rear the next generation (F_2 generation) avoiding the mating of siblings and following an identical procedure as for the F_0 generation. The F_0 and F_1 males and females were killed and necropsied after weaning of their litters. There were no clinical signs or changes in behavior due to treatment. Some differences between controls and treatment groups with regard to body weights and food intake showed no consistent association with the dose or duration of the treatment. In the high-dose group, males of the F_0 and F_1 generation consumed between 4.9-12.4 g trehalose per kg body weight per day during the pre-mating period. F_0 and F_1 females of the high-dose group consumed 5.9-11.7 g/kg bw/d during the same period. During the gestation and lactation period, their trehalose intake ranged from 4.4-7.2 and 10.1-19.9 g/kg bw/d, respectively. No effects of the trehalose treatment were observed on standard parameters of reproductive performance. The number of pups delivered, pup mortality, body weights and growth did not differ between treated groups and controls. No treatment-related macroscopic changes were observed at autopsy. Microscopic examination of reproductive organs and accessory glands, as well as the pituitary and spleen did not reveal any changes that could be attributed to the treatment. It was

concluded that ingestion of trehalose at dietary concentrations of up to 10% had no adverse effects on reproduction and development of the pups (Wolterbeek & Waalkens-Berendsen, 1999a).

Rabbits

In a study of embryotoxicity and teratogenicity, groups of 16 presumed pregnant New Zealand white rabbits were fed diets containing trehalose (purity: 99%) at concentrations of 0, 2.5, 5, or 10% on days 0-29 of gestation. The animals were examined throughout the study, and body weights and food consumption were recorded regularly. The animals were killed on day 29 of pregnancy, and the fetuses were examined for signs of toxicity, external malformations, and soft-tissue defects and were stained for detection of skeletal anomalies.

Body weights, weight gains and food consumption did not differ between controls and treated groups. However, there were a few animals in each of the three dose groups, which consumed only small amounts of food during the third and/or fourth week of pregnancy. One of these animals was killed in extremis, another died spontaneously (both belonging to the high-dose group). At necropsy, a hairball was found in the stomach of both animals. The trehalose intake ranged from 0.21-0.77, 0.48-1.34, and 1.04-2.82 g/kg bw/d for the low-, mid- and high-dose groups, respectively. Necropsy on the does showed no adverse effects that could be related to the treatment. The number of viable litters, the number of corpora lutea, and the mean number of implantation sites were similar in all groups. One early delivery occurred in the high-dose. Fetal length and body weight were similar in all groups. Examination of the fetuses revealed no treatment-related increase in gross, skeletal, or visceral abnor-

malities. Under the conditions of this assay, trehalose was not teratogenic and did not induce and maternal or developmental toxicity (Wolterbeek & Waalkens-Berendsen, 1999b).

9.3.5. Special studies

9.3.5.1. Ocular irritation

A single ocular application of a 10% aqueous solution of trehalose was not irritating or corrosive to the eyes of albino rabbits (Atkinson & Thomas, 1994f).

9.4. Tolerance of trehalose in humans

9.4.1. Intestinal tolerance in healthy volunteers

Being rapidly digested to glucose, trehalose is expected to be tolerated at even high oral doses. Indeed, abdominal symptoms or diarrhea were not reported in a study in which 60 healthy subjects ingested a solution of 50 g trehalose in 400 ml water after an overnight fast (Bolte et al., 1973). The ingestion of a single dose of 25 g trehalose dissolved in 200 ml water 1 hour after breakfast also did not provoke diarrhoea or other noteworthy abdominal symptoms in any of the ten test subjects (Heine et al., 1996).

Clinical signs of intolerance were not observed in any of 27 infants and children (10 healthy and 17 with malabsorption) after ingestion of a bolus trehalose dose of 2 g/kg bw (Fiehring et al., 1976). This observation is consistent with fact that the intestinal trehalase activity is fully developed already during the first year of life (Niessen et al., 1975).

In order to determine the laxative threshold of trehalose, 20 Japanese female students received trehalose solutions (200 ml) about 2-3 hours after a meal. The trehalose content of the solution was increased gradually from 10 to 20, 30, 40, 50 and 60 g. Lactulose was administered according to the same procedure as a positive control. A negative control (e.g., glucose) was not included in the study. A trehalose intake of 0.65 g/kg bw represented the maximum dose at which there was no occurrence of very soft ("muddy") or watery stool. Other signs of malabsorption such as flatulence, borborygmi and distention were reported to occur at doses of $\geq$ 30 g. Surprisingly, the incidence of these symptoms was only slightly smaller after trehalose than after lactulose ingestion. In the absence of a negative control treatment, it cannot be decided to what extent an over-reporting of intestinal symptoms may have contributed to this result (Oku & Okazaki, 1998).

An intake of 10 g trehalose did not increase breath hydrogen expiration and did not elicit any gastrointestinal symptoms in 30 healthy volunteers (10 Japanese, 10 Caucasian, 8 Black, and 2 others). At doses of 20 g or more, mild gastrointestinal symptoms (type not specified) were noted and hydrogen expiration was increased. When the subjects received a single trehalose dose of 0.6 g/kg bw, 90% of the Japanese volunteers reported gastrointestinal symptoms while 11%

of the Caucasians and none of the Blacks appeared to be affected. Blood glucose levels rose to a significantly smaller degree in Japanese subjects than in Caucasians or Black. This result suggests that the rate of trehalose digestion is lower in Japanese people than in Caucasians or Black (Ushijima et al., 1995).

9.4.2. Impaired intestinal tolerance due to intestinal trehalase deficiency

A relative or absolute deficiency of intestinal trehalase results in an intolerance to trehalose ingested in high amounts. Only a few cases of primary (i.e., hereditary) or secondary (i.e., acquired)[27] trehalase deficiency have been reported in the scientific literature.

A first case of trehalose deficiency was observed in Switzerland by Bergoz in 1971. The patient, a 71-year old women, had noticed that the intake of mushrooms ("Champignons de Paris") provoked regularly diarrhoea before the end of the meal. When the patient was subjected to a trehalose tolerance test (ingestion of 50 g trehalose), the typical abdominal symptoms of carbohydrate malabsorption, including diarrhoea, developed promptly. Blood glucose levels did not rise after trehalose ingestion. It was concluded that a relative or absolute deficiency of intestinal trehalase was the cause of the observed symptoms.

[27] A generalized deficiency of disaccharidases (including trehalase) occurs not infrequently in patients who suffer from various disorders of the digestive tract including Morbus Crohn, Colitis ulcerosa, Sprue, etc. (Mališ et al., 1972; Bolte et al., 1973; Berg et al., 1973; Lutkic et al., 1985; Rodeck & Dominick, 1983; Gupta et al., 1999). The reason is that in these subjects the integrity of the intestinal mucosa is impaired generally.

A second, similar case was reported two years later from a clinic in Czechoslovakia (Madzarovová-Nohejlova, 1973). In this case, the absence of intestinal trehalase could be confirmed in a biopsy taken from the upper jejunum. In addition, the patient's father was also found to be trehalase-deficient.

Two more subjects with a relative trehalase deficiency were identified in a survey of the disaccharidase activities of 100 consecutive, morphologically normal intestinal biopsies (Bergoz et al., 1982).

Since trehalase intolerance may be experienced as intestinal intolerance to mushrooms, the trehalose tolerance was examined in 30 Finnish subjects who were selected for self-proclaimed intestinal mushroom intolerance. For comparison, 34 mushroom-tolerant subjects were included in the study as well. A trehalose tolerance test was conducted with ingestion of a 25-g dose of trehalose (consumed with 400 ml water after an overnight fast) followed by measurement of the rise in blood glucose levels and determination of breath hydrogen and methane. In addition, trehalase activity was measured in duodenal biopsies. A placebo treatment (e.g., with glucose) which would have allowed for a double-blind study design, was not included. In the mushroom intolerant group, 13 subjects reported flatulence and abdominal distention, and 6 experienced diarrhoea. However, a relative trehalase deficiency (by biopsy measurement) was detected in 2 subjects only. In the mushroom-tolerant group, no abdominal symptoms were reported after trehalose intake. The duodenal trehalase-to-sucrase ratio was significantly ($p = 0.03$) lower in the 19 subjects who reported intestinal symptoms than in those who did not experience side-effects (43 subjects). However, the duodenal trehalase activity (expressed in IU/g protein) and the rise in blood glucose af-

ter trehalose intake did not differ between the subjects with and without intestinal symptoms (Arola et al., 1999). In the absence of a double-blind, placebo-controlled design it cannot be excluded that an over-reporting of intestinal side effects in the mushroom intolerant group has contributed to the seemingly more frequent occurrence of trehalose maldigestion.

A relative high incidence of trehalase deficiency has been observed in Greenland Eskimos (2 out of 19 subjects) (Dahlqvist, 1974). In this group of people, deficiencies of lactase (17/19), sucrase (3/19), and isomaltase (3/19) occurred with a high incidence as well. A similarly high prevalence of relative trehalase deficiency (in association with deficiencies of all the other intestinal disaccharidases) was found in an examination of 97 surgical Greenlandic patients. Trehalase activities below the normal range were detected in the small intestinal biopsies of 14 out of 97 subjects. In three of these subjects, the trehalase deficiency was confirmed by a trehalose tolerance test (no increase of blood glucose levels following ingestion of 50 g trehalose) (Gudmand-Høyer et al., 1988, 1996).

It is difficult to derive an estimate on the incidence of clinically relevant trehalase deficiency in the human population from these studies. Disregarding the possible higher incidence of trehalase deficiency in specific populations (Eskimos), the incidence of 2% reported by Bergoz (1982) appears high in view of the data by Welsh (1978) and Gudmand-Høyer (1988) who did not observe a single case of intolerance in 123 American and 248 Danish subjects, respectively. In a more recent investigation in the U.K. of 369 patients with suspected malabsorption, one person had a intestinal trehalase activity below the normal range which was suggestive of primary trehalase deficiency (Murray et al., 2000). In the tolerance studies summarized

in chapter 9.4.1, more than 100 healthy subjects, including children, ingested trehalose at doses of up to 25-30 g without the occurrence of gastrointestinal symptoms (Bolte et al., 1973; Arola et al., 1999; Heine et al., 1996; Pointner et al., 1974; Fiehring et al., 1976).

10. Summary

Trehalose is a naturally occurring disaccharide which consists of two glucose molecules linked in 1,1-position by an α-glycosidic bond.

Its sweetness relative to that of sucrose is about 40-45%. Trehalose is stable under all pH and temperature conditions that are typically encountered in food processing. Being a non-reducing sugar, trehalose does not undergo Maillard reactions. The nutritional properties of trehalose correspond to those of maltose. Both sugars are hydrolyzed to glucose by glucosidases of the small intestinal brush-border membrane. The resulting glucose is absorbed.

Trehalose occurs widely in nature. It is produced in bacteria and yeast cells, fungi, algae, and a few higher plants. In many insects it is produced as a major blood sugar and as a reserve carbohydrate during periods of dehydration and freezing. Trehalose also occurs at low concentrations in a number of foods which are consumed as part of a regular diet (mushrooms, bread, fermented beverages, honey).

The Advisory Committee on Novel Foods and Processes (ACNFP) assessed the safety of trehalose, extracted from yeast, in 1990. The intended food applications that were considered by the Committee at that time, included the use of trehalose for the stabilization of certain foodstuffs during the drying process and upon rehydration (e.g., milk powder, dry soups). On advice of the ACNFP, the Committee on Toxicity (COT), and the Food Advisory Committee (FAC), trehalose was

accepted for use in foods (except infant formulae and follow-up milks) in April 1991 (MAFF, 1991; ACNFP, 1990, 1991).

Hayashibara Co., Ltd., Okayama (Japan) has developed a novel enzymatic process for producing trehalose from food-grade starch on a commercial scale. The two crucial enzymes in the process are maltooligosyl trehalose synthase and maltooligosyl trehalose trehalohydrolase. Both are obtained from a strain of *Arthrobacter ramosus*. This microorganism is generally regarded as nonpathogenic and nontoxigenic. Additional enzymes used in the process are isoamylase from a strain of *Pseudomonas amyloderamosa*, cyclodextrin glucanotransferase (CGTase) from a strain of *Bacillus stearothermophilus*, glucoamylase from a strain of *Aspergillus niger* and α-amylase from a strain of *Bacillus subtilis*. The isoamylase preparation has been subjected to standard Ames tests. An acute toxicity test and a subchronic (90-day) toxicity study in rats were conducted as well. The isoamylase preparation was found to not be mutagenic or toxic under the conditions of these experiments. The safety of all other enzymes and source organisms has been evaluated by JECFA earlier already.

Trehalose produced according to this process, which includes different purification steps, has a purity of > 98%. HPLC analysis revealed the presence of three glycosidic by-products (glucose, glycosyltrehalose, α-D-isomaltosyl-α-D-glucoside).

Trehalose has been used in foods in Japan (since 1995), South Korea and Taiwan. It is used for moisture retention, lower sweetness (in comparison to sucrose), and advantageous properties during drying, freezing and dehydration. Japanese foods formulated with trehalose include certain types of confectionery, cakes, cookies, fruit pu-

rees, desserts, health or sport drinks, as well as rice and noodles. The intake of trehalose with these foods varies between 1-10 g per serving. In a few cases (e.g., candies, certain Japanese confectionery) it may reach 20 g per serving. It is estimated that trehalose will be used in Western foods for the same technological reasons but that the type of foods may differ somewhat. Calculation of the intakes which may result from the anticipated uses in Western food indicates that not more than 8.1 g trehalose will be consumed per eating occasion by the average user of such products (18.6 g by the 90^{th} percentile consumer). The estimated average daily intake of trehalose from all its uses combined is less than 8 g.

The metabolism of ingested trehalose resembles that of maltose (or starch) in that both products are absorbed in the form of glucose. Only a very small fraction of trehalose, if any, may be absorbed as such. In humans, trehalose entering the blood circulation would be eliminated by trehalase in the liver, kidneys and plasma.

The safety of trehalose has been examined in acute and subchronic toxicity tests in mice, rats and dogs using oral and intravenous administration of the test substance. Trehalose produced by the enzymatic process was examined in tests for mutagenicity, chromosomal aberration, and micronucleus formation, embryotoxicity/teratogenicity studies in rats and rabbits, a 2-generation reproduction study in rats, and a 3-month toxicity study in mice. The results of these studies show that trehalose is not genotoxic. Trehalose is generally well tolerated even if ingested at high doses, and is not toxic. Trehalose does not impair reproductive performance and is not embryotoxic or teratogenic. Long-term toxicity/carcinogenicity studies were not conducted with trehalose. However, considering the known

metabolism of trehalose, the high purity of the substance, and the available toxicological data, it would appear that the safety assessment can be concluded even in the absence of chronic studies.

There are numerous data on the intestinal tolerance of trehalose in human volunteers. Depending upon individual sensitivity and possibly race, single doses of 20-50 g are usually tolerated without abdominal side-effects. At higher levels, the presence in the gut of osmotically active, incompletely absorbed carbohydrate manifests itself by abdominal symptoms, such as nausea, borborygmi, colic, and laxation. Qualitatively these effects are similar to those which are known for polyols. However, substantially higher amounts of trehalose are required to elicit such effects except in subjects who are deficient in intestinal trehalase. The incidence of hereditary and acquired trehalase deficiency appears to be very low. In the Western population it is likely to be far below 1%[28].

In conclusion, there is a substantial body of evidence to support the safety of trehalose produced by a novel enzymatic process as a food ingredient. On basis of the available toxicological data and in consideration of the close similarity between the metabolism of trehalose and maltose, it is concluded that trehalose does not present a significant risk for human health at the intake which would result from its intended uses in food.

[28] A higher incidence may be found in subjects who suffer from intestinal disease and in Eskimos. However, these people are likely to be aware of their intolerance to sweet foods because they exhibit a more general disaccharidase deficiency, i.e., they are also deficient in lactase and sucrase/isomaltase.

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Note. Reports printed in italics have been submitted to the ACNFP but are not made public because they either do not contain data that are pivotal for the safety assessment of trehalose produced by the enzymatic process and/or are not required by the guidelines accompanying the Novel Foods Regulation.

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Table 1: Applications of trehalose and maximum levels of use

[The data contained in this table are related to marketing know-how for which the applicant claims confidentiality]

Table 2 Estimated intake of trehalose from the combined proposed uses in food[1)]

Age Group	Eating occasion	Intake per user (g) mean	Intake per user (g) 90th perc.
Children (age 2-12 years)			
	Per Day	5.2	10.9
	Breakfast	3.6	5.8
	Lunch[2)]	4.1	7.8
	Dinner[3)]	4.8	9.2
	Snack	3.7	7.5
	Other occasions	5.8	11.9
Teenagers (age 13-19 years)			
	Per Day	7.5	15.2
	Breakfast	5.1	10.2
	Lunch[2)]	6.6	12.5
	Dinner[3)]	7.1	17.3
	Snack	5.2	9.9
	Other occasions	5.9	10.5
Adults (age ≥ 20 years)			
	Per Day	7.2	16.4
	Breakfast	3.9	7.6
	Lunch[2)]	6.1	15.0
	Dinner[3)]	8.1	18.6
	Snack	5.5	11.9
	Other occasions	9.7	16.5

[1] Based on USDA CSFII 1994-96 data, chewing gum intake not included
[2] Lunch combines reported consumption at both lunch and brunch
[3] Dinner combines reported consumption at both dinner and supper

Table 3: Acute toxicity of trehalose

Species	Sex	Route	LD_{50} (g/kg bw)	Reference
Mouse	Male and female	oral	> 5	Atkinson & Thomas (1994 a)
	Male and female	intravenous	> 1	Atkinson & Thomas (1994 a)
Rat	Male and female	oral	> 16	Mc Rae (1995)
	Male and female	oral	> 5	Atkinson & Thomas (1994 b)
	Male and female	intravenous	> 1	Atkinson & Thomas (1994 b)
Dog	Male	oral	> 5	Atkinson & Thomas (1994 c)
	Male	intravenous	> 1	Atkinson & Thomas (1994 c)

Table 4 : Results of assays for the genotoxicity of trehalose

End-point	Test object	Concentration	Result	Reference
Bacterial gene mutation	*S. typhimurium* TA 1535, TA 1537, TA 98, TA 100; *E.coli* WP2 *uvr A trp*	312-5000 µg/plate	Negative [a]	Kitching, 1995
Chromosomal aberration [b]	Chinese hamster ovary (CHO) cells	312-5000 µg/plate	Negative [a]	Winegar, 1997
Micronucleus formation [c]	Swiss-Webster mouse bone marrow	1250-5000 mg/kg bw (i.p., single dose)	Negative	Winegar, 1997

[a] With and without exogenous metabolic activation (MA)

[b] Two assays were conducted: treatment times 3 h (with and without MA); 3 h (with MA) and 21 h (without MA). Harvest times, 21 and 45 h

[c] Harvest times, 24 and 48 h

TREHALOSE

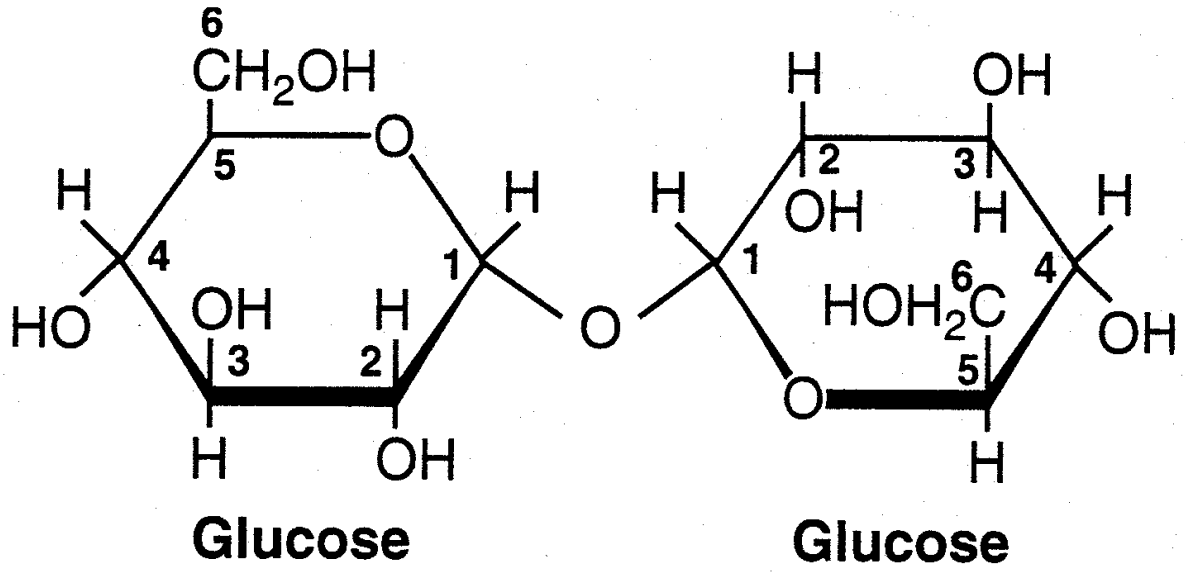


Figure 1: Trehalose is a disaccharide consisting of 2 glucose moieties linked by a glycosidic bond that joins their anomeric carbons.

Figure 2: Action of trehalose producing enzymes

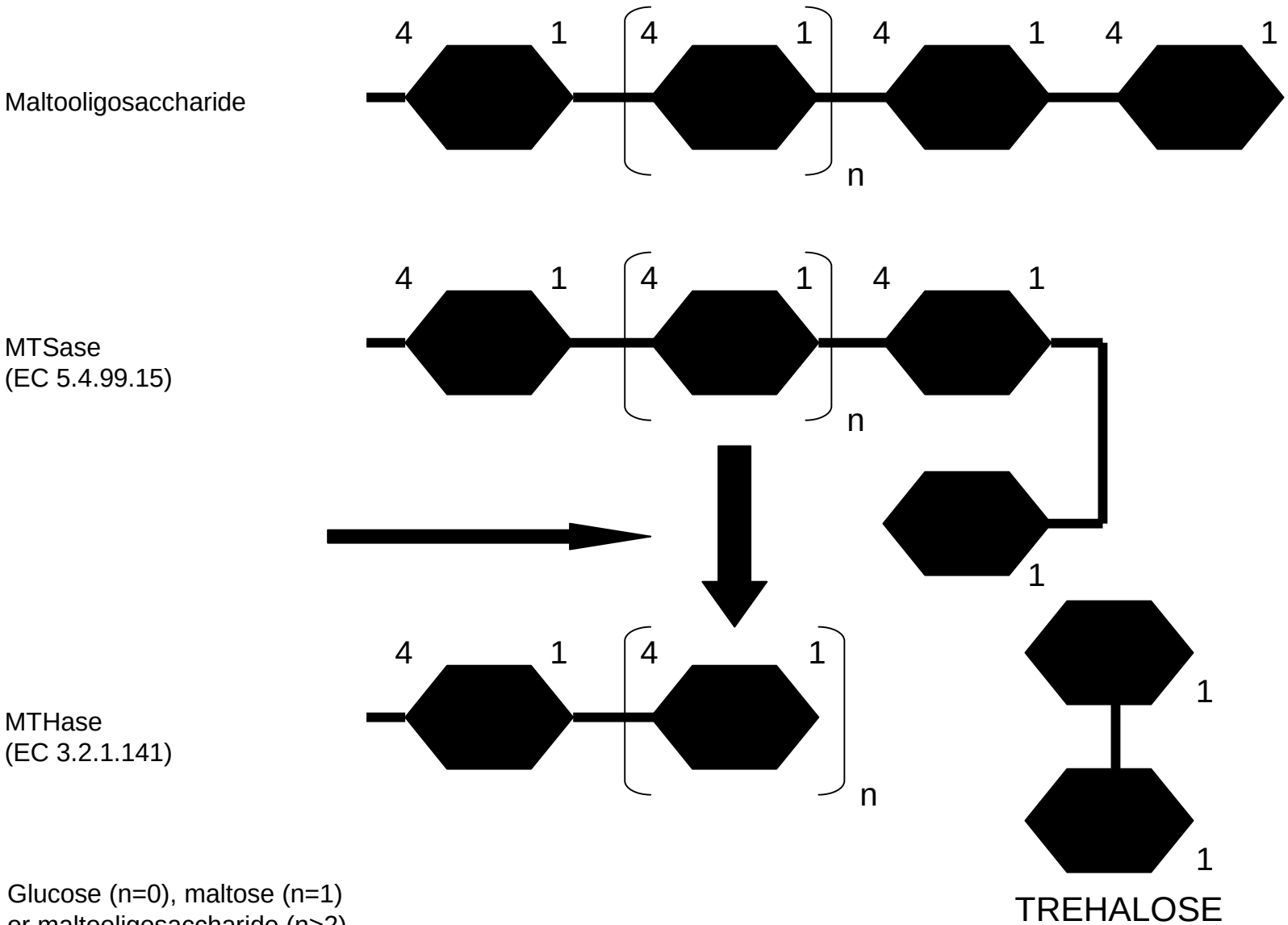
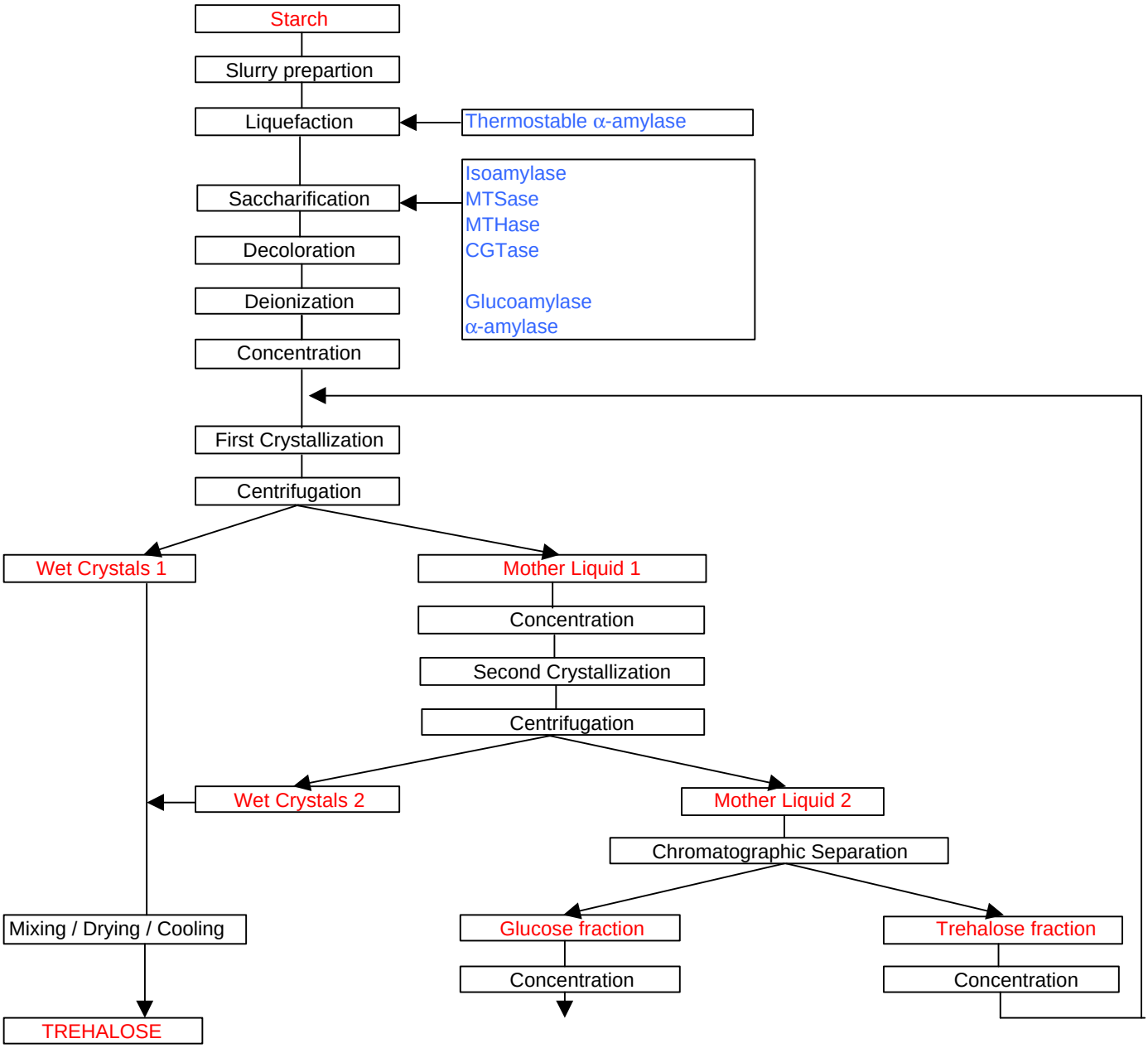


Figure 3: Flow chart of trehalose production



Annex 1

Data requirements for the evaluation of trehalose produced by a novel process (according to Commission Recommendation 97/618/EC and ACNFP decision tree)

Company: Bioresco Product: Trehalose

You have identified the novel food as belonging to:

Class 6 - a food produced using a novel process.

You therefore need to supply information to satisfy the following structured schemes:

I. Specification of the Novel Food
II. Effect of the production process applied to the NF
III. History of the organism used as the source of the NF
IX. Anticipated intake/extent of use of the NF
X. Information from previous human exposure to the NF or its source
XI. Nutritional information on the NF
XII. Microbiological information on the NF
XIII. Toxicological information on the NF

Select OK to continue.

Company: Bioresco Product: Trehalose

Scheme I - Specification of the NF

There is sufficient information from scheme I.

Detailed outcome:

Depending on the derivation and composition of the NF, is appropriate analytical information available on potentially toxic inherent constituents, external contaminants and nutrients?

YES

Is the information representative of the NF when produced on a commercial scale?

YES

Is there an appropriate specification (including species, taxon etc. for living organisms) to ensure that the NF marketed is the same as that evaluated?

YES

OUTCOME: All appropriate analytical information and the appropriate specification of the NF needs to be forwarded with your application.

Company: Bioresco Product: Trehalose

Scheme II - Effect of the production process applied to the NF

The result of scheme II is:

There is sufficient information from scheme II

Detailed outcome:

Does the NF undergo a production process?

YES

Is there a history of use of the production process for the food?

NO

Does the process result in a significant change in the composition or structure of the NF compared to its traditional counterpart?

NO

OUTCOME: Provide all relevant information on the production process.

Company: Bioresco Product: Trehalose

Scheme III - History of the organism used as the source of the NF

The outcome of scheme III is:

Sufficient information from scheme III.

Detailed outcome:

Is the NF obtained from a biological source, i.e. a plant, animal or microorganism?

NO

OUTCOME: No information needed under this scheme.

Company: Bioresco Product: Trehalose

Scheme IX - Anticipated intake/extent of use of the NF

The result of scheme IX is:

There is sufficient information from scheme IX.

Detailed outcome:

Is there information on the anticipated used of the NF based on its properties?

YES

OUTCOME: You will need to provide all relevant information.

Company: Bioresco Product: Trehalose

Scheme X - Information from previous human exposure to the NF or its source

The result of scheme X is:

There is sufficient information from scheme X.

Detailed outcome:

Is there information from previous direct, indirect, intended or unintended human exposure to the NF or its source which is relevant to the Community situation with respect to production, preparation, population, lifestyles and intakes?

YES

Is there information to demonstrate that exposure to the NF is unlikely to give rise to nutritional, microbiological, toxicological and /or allergenicity problems?

YES

OUTCOME: You will need to provide all relevant information. Schemes XI, XII and XIII discuss requirements for nutritional, microbiological and toxicological information.

Company: Bioresco Product: Trehalose

Scheme XI - Nutritional Information on the NF

The result of scheme XI is:

There is sufficient information from scheme XI.

Detailed outcome:

Is there information to show that the NF is nutritionally equivalent to existing foods that it might replace in the diet?

YES

OUTCOME: You will need to provide all relevant information to show the NF is nutritionally equivalent to existing foods that it might replace in the diet.

Company: Bioresco Product: Trehalose

Scheme XII - Microbiological Information on the NF

The result of scheme XII is:

There is sufficient information from scheme XII.

Detailed outcome:

Is the presence of any microorganisms or their metabolites due to the novelty of the product/process?

YES

Is there information to show that the NF is unlikely to contain microorganisms and/or their metabolites of adverse public health significance?

YES

OUTCOME: You will need to provide all relevant information on these microorganisms and/or their metabolites.

Company: Bioresco Product: Trehalose

Scheme XIII - Toxicological information on the NF

There is sufficient information from scheme XIII.

Detailed outcome:

XIII TOXICOLOGICAL INFORMATION ON THE NOVEL FOOD

Is there a traditional counterpart to the NF that can be used as a baseline to facilitate the toxicological assessment?

YES

Compared to the traditional counterpart, does the NF contain new toxicants or changed levels of existing toxicants?

NO

Is there information which suggests that the NF might pose an allergenic risk to humans?

NO

OUTCOME: There is a traditional counterpart to the NF that can be used as a baseline to facilitate the toxicological assessment, and compared to that baseline the NF does not contain new toxicants or changed levels of existing toxicants.

Any allergenic risk to humans from the NF should also be investigated, and the information presented. Controlled allergenicity trials may need to be undertaken where necessary.

All studies and any cited literature references which support the answers to the above questions to establish the toxicological safety of the NF should be forwarded with your application.

Annex 2

Draft specifications

Trehalose

SYNONYMS α,α-trehalose

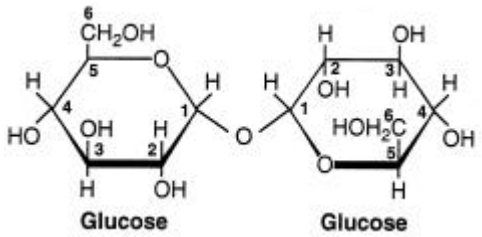
DEFINITION A disaccharide which consists of two glucose moieties linked by a 1,1-glucosidic bond. It is obtained from liquefied starch by a multi-step enzymatic process.

Chemical name α-D-glucopyranosyl-α-D-glucopyranoside

C.A.S. number 99-20-7 (anhydrous)
6138-23-4 (dihydrate)

Chemical formula $C_{12}H_{22}O_{11}$ (anhydrous)
$C_{12}H_{22}O_{11} \cdot 2H_2O$ (dihydrate)

Structural formula



Formula weight 342.30 (anhydrous)
378.33 (dihydrate)

Assay Not less than 98% on an anhydrous basis

DESCRIPTION Virtually odorless, white or almost white crystals (dihydrate) with a sweet taste

FUNCTIONAL USES Bulk sweetener, texturizer, stabilizer, humectant, formulation aid

CHARACTERISTICS

IDENTIFICATION

Solubility	Freely soluble in water, very slightly soluble in ethanol
Specific rotation	$[\alpha]_D^{20}$: +199° (5% aqueous solution of dihydrate)
Melting point	97°C (dihydrate) 210.5°C (anhydrous)

PURITY

Loss on drying	Not more than 1.5% (60°C, 5 h) (Crystal water of dihydrate is not released under these conditions)
Total ash	Not more than 0.05%
Lead	Not more than 1mg/kg Prepare a sample solution as directed for organic compounds in the Limit Test and determine by atomic absorption spectroscopy
Microbiological criteria	Total (aerobic) plate counts: < 300/g Coliforms: Negative by test Salmonella: Negative by test Yeast and molds: < 100/g

METHOD OF ASSAY

Principle: Trehalose is identified by *liquid chromatography* and quantified by comparison to a reference standard containing standard trehalose.

Preparation of sample solution: Weigh accurately about 3 g of dry sample into a 100-ml volumetric flask and add about 80 ml of purified, deionized water. Bring sample to complete dissolution and dilute to mark with purified deionized water. Filter through a 0.45 micron filter.

Preparation of standard solution: Dissolve accurately weighed quantities of dry standard reference trehalose in water to obtain a solution having known concentration of about 30 mg of trehalose per ml.

Apparatus: Liquid chromatograph equipped with a refractive index detector and an integrator recorder.

Conditions:

Column: Shodex Ionpack KS-801 (Showa Denko Co.)

-length	:	300 mm
-diameter	:	10 mm
-packing	:	Shodex Ionpack KS-801
-temperature	:	50°C

Solvent: Water

Flow rate: 0.4 ml/min

Injection volume: 8 µl

Procedure: Separately inject equal volumes of the sample solution and the standard solution into the chromatograph. Record the chromatograms and measure the response of trehalose peak. Calculate the quantity, in

mg, of trehalose in 1 ml of the sample solution by the following formula:

$$125 \times C \times \frac{R_U}{R_S}$$

in which C is the concentration, in mg per ml, of trehalose in the standard preparation, R_U is the peak response of trehalose in the sample preparation, and R_S is the peak response of trehalose in the standard preparation.

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Annex 3

Analytical data of representative batches of trehalose

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Annex 4

Critical Control Points and Control Standards of the production process

Specifications of applied raw material, process chemicals, enzymes and ion exchange resins

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Annex 5

Current applications and use levels of trehalose in Japanese foods

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Annex 6

Projection of trehalose intake by the dietary survey approach