ADVISORY COMMITTEE ON NOVEL FOODS AND PROCESSES

UK/2000/001

Opinion on an application under the Novel Food Regulation from Bioresco Ltd for clearance of Trehalose produced by a novel enzymatic process

Applicant: **Bioresco Ltd (on behalf of Hyashibara Co Ltd)**

Responsible person: **Dr Albert Bar**

Novel Food: **Trehalose produced by a novel enzymatic process**

EC Classification: **6**

Introduction

1. An application was submitted to the UK Competent Authority on 25 May 2000 by Bioresco Ltd (on behalf of Hyashibara Co. Ltd) for approval of trehalose produced by a novel enzymatic process. In 1990, the Advisory Committee on Novel Foods and Processes (ACNFP), the UK Competent Assessment Body, assessed an application, submitted under the voluntary scheme that then existed in the UK for the assessment of novel foods and processes, for the safety of trehalose extracted from yeast. Trehalose produced from yeast was approved for use in foods (except infant formulae and follow-on formulae) in April 1991, although there is no evidence that this product was subsequently marketed in the Community.

2. Trehalose is a naturally occurring disaccharide that consists of two glucose molecules linked by a 1,1-α-glycosidic bond. Its sweetness relative to that of sucrose is about 40-45%. Hayashibara Co., Ltd. has developed a novel enzymatic process for the production of trehalose. In this process, starch is liquefied using an α-amylase enzyme and this raw material is then converted into trehalose using four other enzymes.

3. The two most important of these four enzymes are produced by a strain of *Arthrobacter ramosus*. One enzyme converts the terminal (reducing) maltosyl unit of maltooligosaccharides to a trehalose unit. The other enzyme hydrolyses the α-1,4 glycosidic bond adjacent to the trehalose unit thereby liberating trehalose. In order to increase the yield of the process, two other enzymes (isoamylase from *Pseudomonas amyloderamosa* and cyclodextrin glucosyltransferase (CGTase) from *Bacillus stearothermophilus*) are used as ancillary enzymes. None of the source micro-organisms of these enzymes is genetically modified.

4. The application was prepared according to the European Commission's guidelines. Trehalose was identified as belonging to class 6 - foods produced using a novel process. The Committee consideration of the data provided is presented according to these requirements.

I. Specification of the Novel Food

Information on this aspect is provided in Chapter 2 of the application dossier. Supplementary information on a number of the aspects of the application was requested by the Committee during their deliberations. The supplementary information, in PDF format, has been placed on the website alongside this Initial Opinion.

5. Detailed specifications for trehalose are given in Chapter 2 of the application dossier. In addition, a specification for trehalose was agreed by the Food and Agriculture Organisation (FAO) when trehalose was considered by the Joint FAO/World Health Organisation Expert Committee on Food Additives (JECFA) earlier this year and to be published in FNP 52 edition 8 (2000). These specifications define that the product should be no less than 98% by dry weight, and are subsequently used as criteria for quality control testing of every batch. Proteinaceous impurities from the starch starting material and the different enzyme preparations used are removed by heat denaturation, followed by treatment with activated carbon and filtration. The activated carbon treatment also removes the majority of the other organic, non-ionic impurities. The ionic impurities are removed by a demineralisation step using cation and anion exchange resins. Any remaining inorganic salts are detected by the test for total ash, with a limit of 0.05%. Glycosidic impurities are removed during the crystallisation step and any excessive residues are detected by the HPLC analysis that forms the basis of the assay for trehalose. The Committee sought further detailed information concerning the operating conditions for the various purification steps to provide reassurance that no undesirable toxic substances would carry through into the trehalose end product. Analytical data in support of this specification were included in the confidential data in Annex 3 of the application dossier.

6. The supplementary information dossier included data from analysis of 5 batches of the stabilised enzyme preparation (Section 2(e)). The applicant has claimed confidentiality for these data and they are not included in the version intended for publication on the Internet, although the full data were considered by the Committee.

Discussion

Following consideration of the information provided by the applicant in the original submission, and the further information provided in the supplementary dossier, the Committee was content that the quality control mechanisms employed during the production process and the post-production processing conditions applied were sufficiently robust to ensure that trehalose produced by the enzymatic process described in the dossier and complying with the JECFA (2000) specification to be published in FNP 52 edition 8 (2000), as agreed by FAO/WHO, is safe for use in food.

II. Effect of the Production Process Applied to the Novel Food

A detailed description of the process can be found in Section 3.3 (p 12-15) of the Company dossier. Critical control points for the process are detailed in Annex 4. The applicant has claimed commercial confidentiality for these data. Additional information on the safety of the MTSase / MTHase enzyme preparation was submitted by the applicant in the supplementary dossier.

Novelty of the Process

7. Trehalose extracted from yeast was the subject of an evaluation by the ACNFP in 1990. No indication was given by the applicant of whether the trehalose previously approved by the Committee was ever marketed in the Community. The subject of the present submission is a novel enzymatic process by which trehalose is produced from food-grade starch. Some of the enzymes used in this production process have not previously been used for food production in the Community. Trehalose produced by this enzymatic process is chemically identical to trehalose extracted from yeast.

General Description of the Process

8. In a first step, starch is liquefied by treatment with a thermophilic α-amylase, and then the obtained maltooligosaccharides are treated concurrently with maltooligosyl trehalose synthase (MTSase), maltooligosyl trehalose trehalohydrolase (MTHase), isoamylase, and cyclodextrin glucanotransferase (CGTase). Isoamylase is used as a debranching enzyme, cleaving α-1,6 glycosidic bonds of the starch molecule. CGTase is added in order to recycle maltose back into the process. Glucoamylase (from *Aspergillus niger*) and α-amylase (from *Bacillus subtilis)* 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 decolourised with activated carbon, filtered using diatomaceous earth and perlite as a filtering aid, de-ionised with ion exchange resins, and concentrated by evaporation. Trehalose is obtained by crystallisation.

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

9. Food grade starch is used as the starting material for the 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 suitable for use in the present process.

10. The thermophilic α-amylase (EC 3.2.1.1), which is used for starch liquefaction, is obtained from *Bacillus licheniformi*s. The α-amylase from this source micro-organism has been examined by JECFA and been given an "ADI not specified". The MTSase (EC 5.4.99.15) and MTHase (EC 3.2.1.141) enzymes, which are crucial for the enzymatic synthesis of trehalose, are obtained from *Arthrobacter ramosus* (strain S34). The genus *Arthrobacter* is widely distributed in nature and is generally considered avirulent. Two batches of the MTSase / MTHase enzyme preparation from *Arthrobacter ramosus* were subjected to standard bacterial mutation tests and also to an acute toxicity test in rats. Detailed reports of these tests were presented to the Committee for consideration. The enzyme preparations were shown to produce no adverse effects in any of the tests.

11. The CGTase (EC 2.4.1.19) enzyme is obtained from a strain of *Bacillus stearothermophilu*s. 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 and has been given approval. The isoamylase (EC 3.2.1.68) enzyme from *Pseudomonas amyloderamosa* has been subjected to a range of toxicity studies, including mutagenicity studies and a 90-day toxicity study in the rat.

12. Glucoamylase (EC 3.2.1.3) enzyme from *Aspergillus niger* and α-amylase (EC3.2.1.1) from *Bacillus subtilis* are used in the last steps of the trehalose production process to degrade remaining oligosaccharides and maltose. The safety of these enzymes and source organisms has been evaluated by JECFA and specifications agreed.

Discussion

Detailed information was supplied describing the novel enzymatic process for producing trehalose, including the critical control points used to ensure that a consistent end product is obtained. The Committee noted that the MTSase and MTHase enzymes have not been evaluated for food use in Europe, and that they are produced by an organism that does not have a history of use in food products. However the Committee accepted that there is no statutory requirement in the EU for the approval of enzymes used as processing aids.

The Committee initially expressed concerns over the reproducibility of production of the enzyme preparations and considered that the possibility that the final trehalose product may contain unknown toxicants derived from the production organism had not been fully addressed. Further information on the proposed quality control and assurance programmes for the production of both the enzyme preparation and trehalose, and the post-fermentation purification steps, was subsequently supplied by the applicant. In addition, the applicant provided details of bacterial mutation tests and acute toxicity test in rats on the enzyme preparations, none of which showed any adverse reactions.

The Committee concluded that the supplementary information supplied by the company fully addressed the concerns that were raised during the initial deliberations on the application. The Committee was satisfied that the trehalose production process was fully controlled to produce a consistent trehalose end product and that the post-fermentation purification processes used would remove any unwanted impurities.

The Committee was satisfied that the detailed processing data provided regarding the enzymes used in the production of trehalose and the toxicological data supporting the safety of trehalose produced in this way, ensured that there were no safety concerns regarding the use of those enzymes preparations that had not been submitted for clearance in their own right for use in the production of trehalose, as described in the application dossier. However, for completeness and particularly if any other used for these enzymes were being considered, the Committee would strongly encourage the applicant to submit those enzyme preparations that have not yet been assessed to JECFA or a similar body for an evaluation of their general food safety.

IX. Anticipated intake / extent of use of the novel food

Information on this section of the application is provided in Chapter 5 of the application dossier, and in Annex 5 and 6.

Intended uses in food

13. Trehalose is 40-45% as sweet as sucrose and 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 reduced.

14. 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 flavour because trehalose is less sweet. 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-catalysed hydrolysis.

15. Trehalose also acts as a stabiliser 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. It also stabilises phospholipid bilayers (such as liposomes) and more complex biological structures.

Current food applications of trehalose in Japan

16. Trehalose is used in Japan mainly in bakery products (cakes, frozen bread dough, cream fillings, toppings), beverages (sports drinks, fruit drinks), hard and soft confectionery, fruit jam, breakfast cereals, rice, and noodles. It is used mainly to reduce sweetness (in bakery products and confectionery), to reduce moisture absorption (in breakfast cereals and certain types of confectionery), to reduce browning reactions (in beverages and certain types of confectionery) and to prevent starch retrogradation (in bakery products and noodles).

Estimated daily intake

17. The estimated daily intake (EDI) of trehalose from its different projected uses in food, but excluding chewing gum, has been calculated for the US population by ENVIRON (Arlington, VA) using the dietary survey approach. Though dietary habits of US and European consumers differ, an EDI calculation on the basis of US data was considered by the applicant to be adequate as the consumption of processed food is rather higher in the US than in the EU. Also, European food intake data are not available for conducting EDI calculations with a similar degree of accuracy.

18. This calculation model relies on food consumption data from the 1994-96 Continuing Survey of Food Intakes by Individuals 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 that contains about 6,000 different categories. For the purpose of the EDI calculation it was assumed that each food (or food component) that may contain trehalose, contained it at the highest feasible concentration. Where trehalose was used in a component of the food (such as 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 it could be used, and for all these food categories combined. Mean and 90th percentile intakes were calculated on per-user basis for children (2-12 years), teenagers (13-19 years) and adults (20+ years).

19. “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 occasion], the intake of trehalose could be calculated per eating occasion. For adults, the estimated exposure to trehalose 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).

20. The Applicant suggested that 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 combined total daily intake from all dietary sources. Considering the different anticipated uses of trehalose, the intake of trehalose is not concentrated at certain eating occasions (such as main meals) but is spread evenly over the day. This is also reflected by the data on estimated daily intakes. The data show 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. Trehalose intakes per eating occasion are similar to the intake from one specific food category in a given age group.

Discussion

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. The intakes per eating occasion are also below the threshold dose for abdominal effects in particularly sensitive subjects [>30 g per eating occasion]. Adverse gastrointestinal side effects from the intended uses of trehalose, therefore, are not expected. Since in some applications trehalose may substitute for polyols, the total intake of low-digestible carbohydrates could even slightly decrease.

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

Information on this aspect of the application is provided in Chapters 6 and 9 of the application dossier.

21. Trehalose occurs in bacteria, yeast (such as *S. cerevisia*e), a wide variety of fungi, algae, and a few higher plants. Intracellular trehalose appears to play an important role in the protection of the cells from dehydration and freezing, as well as from other adverse environmental conditions (heat shock, toxic levels of ethanol, osmotic stress). In addition, trehalose may serve as reserve carbohydrate during periods of carbon starvation.

22. 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, small amounts of trehalose have been found in bread (1.2-1.5 g/kg dry weight), beer (45-240 mg/l), wine (44-129 mg/l), and honey (0.1-2.3 g/100g). 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. Trehalose produced by the enzymatic process described in this dossier became available in Japan for food use in 1995. By 1999, annual sales had reached 16-20,000 tons.

Discussion

The Committee agreed that trehalose itself is not a novel product and would have been consumed as a component of a variety of other foodstuffs.

XI. Nutritional information on the novel food

Information on this section of the application is presented in Chapters 7 and 9 of the applicant's dossier.

23. Ingested trehalose is digested by trehalase in the small intestine to glucose that is readily absorbed. The applicant considers that trehalose has the same physiological energy value and is nutritionally equivalent to glucose or maltose. Implications for individuals with trehalase deficiency are discussed in paragraphs 58-9.

Discussion

The committee was content with the information provided by the applicant.

XII. Microbiological information on the novel food

Information on this aspect of the application is provided in Chapter 8 of the application dossier

24. The enzymes that are used in the novel production process of trehalose are obtained from non-genetically modified strains of *Arthrobacter ramosus, Pseudomonas amyloderamosa, Bacillus stearothermophilus, and Bacillus licheniformis*. In addition to filtration of the enzyme-containing fermentation broths, the trehalose production process comprises several heat-treatment steps. Therefore, the inadvertent presence of micro-organisms in the final product is unlikely and, should it occur, it would be detected by the proposed quality control procedures and dealt with accordingly.

Discussion

The Committee was content with the information provided by the applicant and considered that the quality control procedures described would be adequate to detect any inadvertent microbiological contamination of the trehalose product.

XIII. Toxicological assessment of the novel food

Information on this section of the application is presented in chapter 9 of the company's dossier and Section 1 of the supplementary information dossier.

25. The material used in toxicological studies was 99% pure trehalose, whereas the commercial specification defines material of ≥98% purity. This apparent discrepancy in purity was clarified by the applicant. The former value is a measured amount for the batch(es) used in the studies whilst the value for the general specification is a minimum quality that should be attained from the production process. When trehalose of 99.1% purity was analysed by HPLC, the main impurity was glucose (0.5%), along with α-D-maltosyl- α-D-glucoside (0.3%) and α-D-isomaltosyl- α-D-glucoside (0.1%) (Section 3.5.2 of the original application dossier). This information was reiterated in the additional information supplied by the applicant. Following further consideration the Committee was content that the quality assurance parameters employed during the production process, along the subsequent post-production processing steps used, would ensure that the final trehalose product contains no unknown toxicants.

Subchronic oral toxicity study in mice

26. The company has carried out a subchronic oral toxicity study in mice to OECD (No: 408) guidelines in compliance with GLP. Four groups of 20 male and 20 female NMRI mice per group were fed trehalose in the diet at concentrations of 0 (control), 0.5, 1.5, or 5% for 13 weeks. These dietary concentrations correspond to mean intakes (for both sexes) of 0, 840, 2500, and 8300 mg/kg body weight per day.

27. During the study four males died or were killed *in extremis*, No: 78 (high dose group), 57 (mid dose group) 12 (control) and 16 (control). Animals No. 78 and 12 exhibited a severe reduction in food intake prior to death, the other two males died from other non-treatment related causes.

28. Throughout the treatment period food consumption in males was reduced in the high dose group, often attaining statistical significance, and to a lesser extent in the mid dose group. However, because this reduction in food consumption was apparent from the start of the study it is attributed to the unpalatability of high concentrations of trehalose.

Food consumption in females was unaffected. Body weight gain in males was only slightly retarded in the top two dose groups; body weight gain in females was unaffected.

29. Clinical signs (evaluated daily) and opthalmoscopic evaluation (at start and end of study in control and high dose group) were unaffected by treatment. Blood and urine were sampled at weeks 5, 9 and 13. Haematology parameters were unaffected by treatment. While there were a few significant intergroup differences, these were not considered treatment-related as there was no dose-response. Urinalysis did not reveal any treatment-related effects.

30. There was a significant decrease in plasma bilirubin in high dose males at weeks 5 and 9 and in high dose females at week 5. However, the magnitude of the reduction was slight, within the limits of historical controls, and therefore not considered treatment related. Plasma glucose was elevated in the high dose group in both males and females at all three sampling times, significantly so at weeks 5 and 9 in females. Glucose concentrations were also raised on occasions in the low and mid dose groups. These increases are attributed to the metabolism of trehalose to glucose. Plasma calcium concentrations were significantly reduced in the top two male groups at week 13; however, concentrations were unaffected in weeks 5 and 9 in males and at all time points in females and so these increases are not considered treatment related. Plasma potassium concentrations were only determined at week 13 for which there was a dose-related decrease in both sexes, significantly at mid and high dose groups in males and high dose group in females. While the potassium concentrations in controls were slightly higher than expected, an association with treatment cannot be excluded, though the changes were all within the limits of historical controls. In contrast, in females there was a small dose-related increase in plasma phosphorus concentrations, which was significant at weeks 5 and 9 in the top dose group. This trend was also slightly evident in males at week 13 only and though phosphorus concentrations were also significantly increased in the high dose group at week 5 they were lower than controls on week 9 and thus these findings are not considered treatment related. While there were no treatment-related intergroup differences in aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), and alkaline phosphatase (ALP) activity, extremely high AST, ALT, and LDH activity was measured in one high dose male (No: 72) at week 5. However, these high values were not evident at weeks 9 and 13 in this animal or in any other animal at all times and are therefore not considered treatment related.

31. Adrenals, brain, heart, kidneys, liver, ovaries, pituitary gland, prostate, spleen, testes, thyroid and thymus weights were recorded at necropsy and data for absolute weight and organ:body weight and organ:brain weight ratios presented, none of which were significantly affected by treatment.

32. At necropsy there were no treatment-related gross pathological changes. Representative tissue samples from an extensive number of organs from animals from groups 1 (control) and 4 (high dose) were subject to histological evaluation as were any tissues from groups 2 and 3 exhibiting gross pathological change at necropsy or from any unscheduled deaths (No: 57). There were no treatment-related histological findings. Animal 78, killed *in extremis*, had severe pyelonephritis but this was not considered treatment-related; at necropsy, gravel (a term used by the authors to presumably refer to salt deposits) was detected in the urinary bladder and right kidney. Animal 12 had extreme thoracic inflammation, which the authors conclude contributed to the animal's death.

33. In conclusion, the administration of trehalose to mice for 13 weeks at dietary concentrations of up to 5%, equivalent to 8300 mg/kg body weight per day, was well

tolerated with no evidence of toxicity. Thus, the No Observed Adverse Effect Level (NOAEL) can be considered to be 8300 mg/kg body weight per day.

Oral two generation reproduction study in rats

34. The company has carried out an oral two generation, one litter per generation, reproduction study in rats, to OECD (No: 416) guidelines in compliance with GLP.

35. Four groups of 28 male and 28 female albino rats per group (F_0 generation) were fed trehalose in the diet at concentrations of 0 (control), 2.5, 5, or 10 % for 10 weeks prior to mating, throughout mating and gestation periods and during lactation until they were killed. After weaning, the F_0 male and female parents were killed and subjected to necropsy. The total litter size, sex ratio, number of stillbirths and livebirths, live and dead pups, pup weight, and external abnormalities in the F_1 generation, were recorded on postnatal day (PND) 1, 4, 7, 14, and 21 where appropriate. On PND 4, litters were standardised to 4 males and 4 females per litter. All stillborn pups, pups found dead, or pups killed *in extremis* were subject to necropsy (as were similar pups of the next (F_2) generation). After weaning, 28 male and 28 females from the F_1 generation were randomly selected to rear the F_2 generation (mating of siblings was avoided; animals mated were over 10 weeks old). The non-selected F_1 animals were discarded. The selected F_1 animals were administered trehalose at the same dietary concentrations as the F_0 generation until weaning of the F_2 generation, after which the F_1 parents were killed and subjected to necropsy. The procedures followed to rear the F_2 generation litter were reported to be identical to those used to rear the F_1 generation litter; litter size was standardised on PND 4 and the same set of observations/measurements taken. The following organs from control and high dose F_0 and F_1 animals were subject to histological investigation plus all organs exhibiting gross pathological change at necropsy: ovaries, uterus, vagina, testes, epididymides, seminal vesicles (with coagulating glands and their fluids), prostate, pituitary, and spleen. In addition, the reproductive organs of males that failed to sire and non-pregnant females of the low and mid dose groups were histologically examined. At necropsy the spleen was weighed (there is no justification as to why only the spleen was weighed).

36. Clinical signs (checked at least once daily) during premating, gestation, and through to weaning were unaffected by treatment. While there were a few significant changes in body weight and body weight gain in both the F_0 and F_1 generations, these changes were not consistent and exhibited no dose-response and were therefore not considered to be treatment-related. While food consumption was significantly increased (though decreased on one occasion) on a number of occasions in both generations, primarily during the pre-mating periods, these changes were not consistent and were therefore not considered to be treatment-related. The mean intake of trehalose for F_0 and F_1 males was 1.7, 3.5, and 7.1 g/kg body weight per day in the low, mid and high dose groups respectively. For F_0 and F_1 females, the corresponding intakes during the premating period were 1.9, 3.7, and 7.1 g/kg b.w./day. During the gestation period, the intakes were 1.5, 3.1 and 6.2 g/kg b.w./day and the lactation period were 3.3, 6.9 and 14 g/kg b.w./day.

37. At necropsy of both parental generations, absolute and relative spleen weight were unaffected by treatment. There were very few gross pathological changes, none of which were considered to be treatment-related. There were a number of histological changes, though they occurred with low incidence and without a dose-response and therefore were not considered to be treatment-related.

38. In both generations there were no treatment-related effects on the fertility and reproductive parameters assessed, namely: precoital time, mating index, male and

female fertility, female fecundity index, gestation index, duration of gestation, and post-implantation loss (though no details are provided on how the number of implantation sites were assessed).

39. Trehalose had no consistent adverse effects on litter size, the number of liveborn pups (the number of liveborn pups was significantly increased in the high dose group of the F_0 generation and mid and high dose groups of the F_1 generation), the number of stillborn pups (the number of stillborn pups was significantly decreased in the high dose group of the F_0 generation), sex ratio, pre-cull (days 1-4) pup mortality (pup mortality was significantly decreased in the low and high dose F_0 groups), post-cull pup mortality, and sex ratio. It was reported that no grossly malformed pups were observed and the results of necropsy of stillborn pups, pups that died or were killed *in extremis* did not indicate any abnormal development. There were no treatment-related effects on mean pup body weight and pup body weight changes. Clinical signs in the pups from PND1 to weaning were unaffected by treatment when evaluated on a litter basis in both generations. On an individual pup basis there were a number of significant inter-group differences; however, none of these clinical signs exhibited a dose-response or were consistent across generations and therefore were not considered to be treatment-related.

40. In conclusion, dietary administration of trehalose at dietary concentrations up to 10% had no effects on reproduction of the parental F_0 and F_1 generation or the development of F_0 and F_1 generation pups. Taking the lowest recorded intake of the high dose group, the NOAEL can be considered to be approximately 6g trehalose/kg body weight per day.

Developmental study in rabbits

41. The company has carried out a developmental study in New Zealand white albino rabbits to OECD (No: 414) guidelines in compliance with GLP.

42. Four groups of 16 artificially inseminated female rabbits were administered trehalose in the diet at concentrations of 0 (control), 2.5 % (low), 5 % (mid) and 10 % (high) from gestational day (GD) 0 (day of a.i.) to GD 29. These dietary concentrations correspond to intakes of 0, 540, 1100, and 2000 mg trehalose/kg body weight per day throughout gestation. On day 29 the animals were killed and subject to necropsy. Reproductive organs were weighed and examined. Foetuses from all dose groups were examined for external and visceral abnormalities and, with the exception of foetuses from the low dose group, were also examined for skeletal abnormalities.

43. During the study one high dose animal (No: 113) was killed *in extremis* as it was not eating and another high dose animal (No: 117) was found dead. At necropsy both animals were found to have a hairball in their stomachs. Though the cause of death of No: 117 was unknown, neither the death of No: 117 nor the moribund condition of No: 113 were considered treatment related.

44. Clinical signs in all animals were unaffected by treatment. In those animals that were pregnant there were no significant differences in mean body weights, body weight changes and food consumption. At necropsy, there were no treatment-related gross pathological changes.

45. Twelve, twelve, fourteen, and thirteen animals (including the two dead animals and one animal that underwent an unscheduled early delivery) from the control, low, mid, and high dose groups respectively were found to be pregnant. In those animals pregnant at GD29, the number of corpora lutea, implantations, early/late resorptions, live and dead foetuses, and sex ratio of the foetuses were unaffected by treatment. Carcass and

ovaries weight and net weight change (body weight gain during gestation minus gravid uterine weight) were unaffected by treatment. However, in the high dose group, the gravid uterus weight was higher than controls and this difference was significant when empty uterus weight (i.e. uterus weight minus foetuses and placenta) was compared. The authors attribute this increase to the (non-significant) higher number of foetuses in the high as opposed to the control group (8.4 verses 6.5 respectively). The mean foetal weights and placental weights did not differ significantly between treatment and control groups. Aside from one control foetus with proboscis and ectopic eyes, no external foetal abnormalities were observed. There were no treatment-related placental and visceral foetal abnormalities. With regards to skeletal abnormalities, there was a significant increase in the incidence of accessory ribs in the mid-dose, though not the high-dose, group when expressed on a foetal basis, though not when data for litters were analysed and for these reasons this effect was not considered to be treatment-related. The incidence of unossified distal epiphysis of humerus was significantly increased in the mid-dose group when expressed on a foetal basis, though not when data based on litters were analysed. However, the incidence in the high dose group was not significantly different from controls and thus this effect was not considered to be treatment-related. The incidence of one or two incomplete ossified thoracal bodies was significantly increased, when expressed on a foetal basis, in the mid and high dose groups, though when expressed on a litter basis this was only significant in the mid dose group. However, the incidence of three or more incompletely ossified thoracal bodies was slightly decreased in the mid and high dose groups. Furthermore, the incidence of total incompletely ossified thoracal bodies was only significantly increased when expressed on a foetal basis in the mid-dose group and thus these effects were not considered to be treatment-related.

46. In conclusion, trehalose did not induce maternal or developmental toxicity at concentrations up to 10% in the diet, equivalent to a dietary intake of 2 g trehalose/kg body weight per day throughout gestation.

Developmental study in rats

47. The company has carried out a developmental study in Wistar rats to OECD (No: 414) guidelines in compliance with GLP.

48. Four groups of 28 pregnant rats were administered trehalose in the diet at concentrations of 0 (control), 2.5 % (low), 5 % (mid) and 10 % (high) from gestational day (GD) 0 to GD 21. These dietary concentrations correspond to intakes of 0, 1.7, 3.5, and 6.9 g Trehalose/kg body weight per day throughout gestation. On day 21 the animals were killed and subject to necropsy. Reproductive organs were weighed and examined. Foetuses from all dose groups were examined for external abnormalities and foetuses from the control and high dose groups were also examined for visceral and skeletal abnormalities.

49. There were no unscheduled deaths during the study. Aside from one high dose and one control animal exhibiting haemorrhagic discharge there were no other remarkable clinical observations. At necropsy there were no treatment-related gross pathological changes. In those animals that were pregnant there were no significant differences in mean body weights, body weight changes and food consumption.

50. During the study 22 (including 1 early delivery), 25 (including 2 early deliveries), 24, and 24 females in the control, low, mid, and high dose groups were pregnant respectively. In those animals that were pregnant there were no significant differences in mean body weights, body weight changes and food consumption. In those animals pregnant at GD21, the number of corpora lutea, implantations, early/late resorptions, live

and death foetuses, and sex ratio of foetuses were unaffected by treatment. In these animals there were no significant differences in empty and gravid uterus weight, ovaries weight, carcass, and net weight change. The mean foetal weights and placenta weights did not differ significantly between treatments and controls. The incidence of large foetuses (that is foetal weight >125% of mean foetal body weight) was significantly reduced in the low and high dose groups (though was unaffected in the mid dose group). However, the incidence of small foetuses (that is foetal weight <75% of mean foetal body weight) was also significantly reduced in the low dose group and, though not significantly, also in the high dose group. For this reason and the lack of a dose-response, these effects on foetal size were not considered to be treatment-related. There were no treatment-related external foetal or placental abnormalities. In foetuses from the control and high dose groups that were pregnant at GD21, there were no treatment-related visceral or skeletal abnormalities.

51. In conclusion, trehalose did not induce maternal or developmental toxicity at concentrations of up to 10% in the diet, equivalent to a dietary intake of 6.9 g Trehalose/kg body weight per day throughout gestation.

Genotoxicity data on trehalose

52. Three mutagenicity assays have been submitted by the company, two *in vitro* and one *in vivo* assay, all in compliance with GLP.

Gene mutation in bacteria

53. This study was carried out to OECD guideline Nos: 471 and 472. Four strains of *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537) and a tryptophan-dependent mutant of *Escherrichia coli* were exposed to trehalose (purity: 100%) at test concentrations of 312.5, 625, 1250, 2500 and 5000 µg/plate in two independent experiments. The test concentrations were determined from a dose range finding cytotoxicity study, in the presence and absence of S9 mix of Aroclor 1254 pre-treated rats. Distilled water was used as solvent and negative control. N-Ethyl-N'-nitrosoguanidine and 2-aminoanthracene were used as positive control in the absence and presence of an activation system respectively.

54. No cytotoxicity was observed with any of the test concentrations. The test substance did not induce gene-mutations in any of the tester strains. The positive controls produced the expected increase in mutant frequencies. Therefore, the test substance was not regarded to be mutagenic under the conditions of this test.

Chromosomal aberrations in cultured mammalian cells

55. Trehalose (purity: 99.2%), was tested in the chromosomal aberration assay using Chinese Hamster Ovary cells in the presence and absence of S9 mix in two independent experiments. Sterile water was used as the solvent. In experiment 1 cells were exposed to trehalose for 3 hours in the presence and absence of an activation system. On the basis of the cytotoxicity assay, the concentrations of trehalose tested in the first assay were 1250, 2500 and 5000 µg/ml; metaphase cells were then harvested at 21hr after initiation of exposure. The replicate assay employed the same concentrations of the test substance and cultures were exposed to trehalose for 21hr in the absence of an activation system and for 3hr in its presence. Cultures in the replicate experiment were then harvested at 21hr and in the case of the control and high dose, at 45 hr. The 45-hr cultures were also examined for polyploidy at the 5000 µg/ml dose. Methyl methanesulfonate and cyclophosphamide were used as positive controls in the absence and presence of an activation system respectively.

56. No cytotoxicity was observed in the presence or absence of an activation system when Trehalose was tested up to 5000 µg/ml. Trehalose did not produce chromosomal aberrations or induce polyploidy in either experiment. Therefore, the test substance was not regarded to be clastogenic under the conditions of this test.

Micronucleus assay

57. Trehalose (purity 99.2%) was tested in the mouse bone marrow micronucleus test using male and female mice. The compound was administered by a single intraperitoneal dose to groups of 10 animals per sex. Five mice per sex were sacrificed at 24 or 48 hours post treatment for the assessment of cytotoxicity and micronucleus formation. The compound was dissolved in sterile water and tested at the following doses: 1250, 2500 and 5000 mg/kg body weight. A vehicle control group and a cyclophosphamide positive control group were also evaluated. Two principal parameters were determined using 1 slide/animal 1) the number of polychromatic erythrocytes (PCE) among 200 total erythrocytes (RBC) per animal and 2) the number of micronucleated RNA positive erythrocytes (MPE) per 2000 PCE per animal.

58. There were no clinical signs or early deaths reported. Trehalose produced a negative response in this assay. The positive control produced the expected increase in micronucleated cells. Therefore, the test substance was not regarded as being clastogenic under the conditions of this test.

Human studies

59. Trehalose is rapidly metabolised in the gut to glucose by the brush border enzyme trehalase. A small minority of the population exhibits a primary (hereditary) or secondary (acquired) trehalase deficiency and thus may experience intestinal discomfort such as laxation, after ingestion of excessive amounts of trehalose due to the osmotic activity of undigested trehalose in the gut. However, smaller amounts of trehalose are tolerated by such individuals without any such symptoms. The prevalence of trehalase deficiency is low; the company suggests, on the basis of a limited number of studies, that the prevalence in western populations is rare, much less than that for lactase deficiency (Murray et al British Journal of Nutrition (2000) Vol 83(3) p 241-245).

60. The company has summarised a number of studies investigating intestinal tolerance following single bolus oral doses of trehalose in healthy participants. More than 100 participants are reported to have ingested trehalose at single doses up to 20-30 g without the occurrence of gastrointestinal symptoms. At higher doses gastrointestinal symptoms such as flatulence, watery stool, and distension were reported to occur. In trehalase-deficient individuals such effects are likely to occur at lower trehalose intakes. However, the intake of trehalose that would be tolerated by such individuals is unclear though the severity of any such gastrointestinal effects will also be dose-dependent. Only one study has investigated trehalose tolerance in individuals with self-reported mushroom intolerance as a surrogate of trehalose intolerance. Mushrooms area natural source of trehalose and individuals with trehalase deficiency may only recognise that they are mushroom-intolerant. While the interpretation of this study is limited by its design, mushroom intolerance was not limited to those individuals with trehalase deficiency as determined by trehalase activity of gut biopsies. Furthermore, the rise in plasma glucose concentrations after trehalose intake did not differ between subjects with and without reported gastrointestinal symptoms.

Discussion

A number of animal toxicological studies have been conducted on trehalose produced by this enzymatic process. The administration of trehalose to mice for 13 weeks at dietary concentrations of up to 5%, equivalent to approximately 8g trehalose/kg body weight per day, was well tolerated with no evidence of toxicity. Furthermore, trehalose did not cause maternal nor developmental toxicity in rabbits and rats when administered at dietary concentrations up to 10%, equivalent to approximately 2 and 7 g trehalose/kg body weight per day respectively. In a two-generation reproduction study in rats trehalose had no effects on reproduction and development when administered at dietary concentrations up to 10%, equivalent to 6 g trehalose/kg body weight per day.

Trehalose is a naturally occurring disaccharide (carbohydrate), that consists of two glucose molecules linked by a glycosidic bond. It is metabolised in the gut and is absorbed as glucose. The potential subchronic, reproductive and developmental toxicity of trehalose has been investigated in a number of studies in animals and was well tolerated at dietary concentrations up to 10%. Furthermore, trehalose was not shown to be mutagenic. While no chronic/carcinogenicity studies with trehalose have been conducted, these are not considered necessary in assessing the safety of trehalose in light of the available toxicological data and the fact that trehalose is completely metabolised to glucose.

Predicted dietary intakes of trehalose by the mean and high-level (90th percentile) consumer are estimated as 8 and 19 grams per day respectively. These intakes are lower than the doses of trehalose that were ingested by healthy participants (up to 30g) without the occurrence of gastrointestinal symptoms and thus adverse gastrointestinal effects in the general population from the intended uses of trehalose are not expected. In individuals who are deficient in the enzyme trehalase that breaks down trehalose, and are thus trehalose intolerant, gastrointestinal symptoms may occur at lower intakes. However, trehalose intolerance is estimated to affect <1% of the population.

The Committee noted that some individuals who believe themselves to be intolerant to mushrooms may, in fact, be deficient in the enzyme trehalase. Such individuals would also be intolerant of foods containing significant amounts of trehalose produced by this enzymatic process, although they would be able to tolerate foods containing small amounts of trehalose. However not all individuals with mushroom intolerance are deficient in the enzyme trehalase.

The Committee considered that, given the wide range of toxicological information supplied by the applicant, trehalose produced by this enzymatic process is safe for use within the range of foodstuffs detailed by the company.

OVERALL DISCUSSION

61. The application contains good specification data and a detailed description of the production process. The process is well controlled and consistent product is produced. A specification for trehalose produced by this enzymatic process has been agreed by FAO.

62. There are no nutritional concerns for the product as trehalose is readily converted to glucose. The eating occasion data provided show that there was no glucose overload on the occasions when the trehalose was consumed.

63. The trehalose produced by this enzymatic process has been shown to be non-toxic and non-mutagenic. Many of the proposed uses for trehalose are mutually exclusive and there are sufficient safety factors between the predicted intake of the product and the level tested in experiments. There was a 60x safety margin between the proposed average intake of trehalose and the highest dose tested in animals and a 20x safety margin between the proposed extreme (90th percentile) intake and the highest dose tested.

64. No information was provided in the application dossier concerning proposals for labelling of the product. During their deliberations, members of the ACNFP were concerned that diabetics may be unaware that trehalose is a disaccharide of glucose, and not take it into consideration when managing their dietary calorific intake

65. It was therefore suggested that it might be helpful to include the description 'a sugar' after the name trehalose in the ingredient list, and the Committee therefore sought the advice of the UK Food Advisory Committee on the labelling requirements for trehalose in relation to the needs of diabetics. Taking account of the advice received, the Committee recommends that trehalose should be listed as an ingredient in the foods to which it is added. In addition, trehalose content should be taken into account when determining nutritional labelling information, particularly the content of sugars and carbohydrates in food products, so that diabetics are fully able to manage their overall calorie intake. The Committee was advised that there are no general powers to add a description such as 'a sugar' to the name trehalose in the ingredient list. In addition, under the EC Food Labelling Regulations, the term 'sugar' is a reserved generic description that may only be used for ingredients that are 'any type of sucrose' and may therefore not be used as a description to accompany trehalose. Furthermore, other materials, such as maltose and lactose, that may also be added to a range of food products, are not described in this way, and thus there is no precedent for such additional information on the labels of foods containing trehalose. Nevertheless, the Committee considers that information should be provided to health professionals caring for diabetics and to the relevant support groups, so that diabetics are aware that trehalose is a source of glucose. This approach has been adopted in the past to ensure that diabetics have an above average knowledge of the nutritional quality of food.

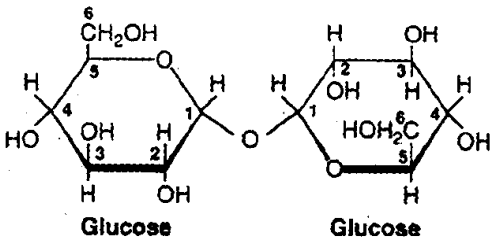
66. The Committee noted that some of the enzymes preparations used to produce trehalose have not been formally assessed for safety in their own right. However, the Committee was satisfied that the detailed processing information provided, together with the range of toxicological data on trehalose produced using this process, provided sufficient reassurance as to their safety for this particular use. However, the Committee agreed that the applicant should be strongly encouraged to submit as soon as possible, for formal evaluation, information on the enzyme preparations used in the trehalose production process that have not yet been evaluated for their general food safety, particularly if other food uses are anticipated/.

CONCLUSION

67. The Advisory Committee on Novel Foods and Processes is satisfied that trehalose produced by the novel enzymatic process described and complying with the specification agreed at the 55th JECFA and to be published in FNP 52 edition 8 (2000) can be approved as a novel food ingredient, to be used in the range of foodstuffs detailed in the application dossier.

Trehalose – Specification (slightly revised specification was agreed at the 55th JECFA (2000) and will be published in FNP 52 edition 8 (2000)

SYNONYMS	α, α - trehalose
DEFINITION:	A non-reducing disaccharide that consists of two glucose moieties linked by an α, 1,1-glucosidic bond. It is obtained from liquefied starch by a multi-step enzymatic process. The commercial product is the dihydrate.
Chemical name	α -D-glucopyranosyl-α-D-glucopyranoside
C.A.S. number	6138-23-4 (dihydrate)
Chemical formula	$C_{12}H_{22}O_{11} \bullet 2H_2O$ (dihydrate)
Structural formula	



Formula weight	378.33 (dihydrate)
Assay	Not less than 98% on an anhydrous basis.

DESCRIPTION Virtually odorless, white or almost white crystals with a sweet taste.

CHARACTERISTICS

IDENTIFICATION

Solubility Freely soluble in water, very slightly soluble in ethanol.

Specific rotation $[\alpha]_D^{20}$ +199° (5% aqueous solution).

Melting point 97°C (dihydrate)

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, appropriate to the specified level.

Microbiological criteria
Total (aerobic) plate counts: < 300/g
*Coliform*s: 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 integrating 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: Inject separately equal volumes of the sample solution and the standard solution into the chromatograph. Record the chromatograms and measure the size of response of the trehalose peak.

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

$$\%\text{trehalose} = 100 \times (R_U / R_S)\ (W_S / W_U)$$

where

R_S = peak area of trehalose in the standard preparation
R_U = peak area of trehalose in the sample preparation
W_S = weight in mg of trehalose in the standard preparation
W_U= weight of dry sample in mg.