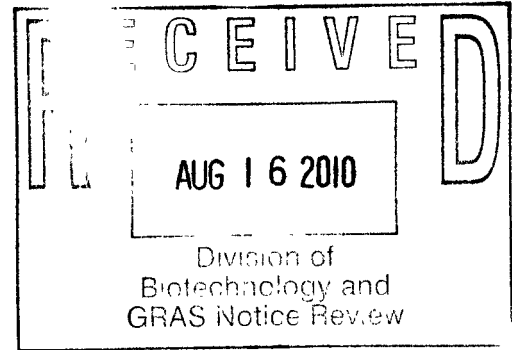


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ORIGINAL SUBMISSION

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August 12, 2010

Dr. Robert Martin
Division of Biotechnology and GRAS Notice Review
Office of Food Additive Safety-CFSAN
U.S. Food and Drug Administration
5100 Paint Branch Parkway (HFS-255)
College Park, MD 20740-3835

Re: GRAS exemption claim for D-tagatose as an ingredient in foods

Dear Dr. Martin,

This is to notify you that CJ Cheiljedang (based in S. Korea) claims that the use of the substance described below (D-tagatose) is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act because CJ America has determined such use to be Generally Recognized As Safe (GRAS).

On behalf of CJ Cheiljedang, NutraSource (an independent consulting firm) assembled a panel of experts highly qualified by scientific training and experience to evaluate the safety of the intended uses of D-tagatose. The panel included Dr. Susan Cho at NutraSource (Clarksville, MD), Dr. James Anderson (University of Kentucky, Emeritus, Lexington, KY), and Dr. George Fahey at the University of Illinois (Urbana, IL). Following independent critical evaluation of the available data and information, the panel has determined that the use of D-tagatose (that is manufactured by CJ CheilJedang, S. Korea) described in the enclosed notification is GRAS based on scientific procedures.

After reviewing the available data, the Expert Panel concluded in its August 2010 statement that the intended use of CJ Cheiljedang's D-tagatose (to be used as in ingredient in foods ready-to eat breakfast cereals, diet soft drinks, non-diet soft drinks, confectionery, formula diets for meal replacement, meal replacement drink mix (powder), cake, pie, cake mix powder, frostings, ice cream and frozen yogurt, yogurt, frostings, sugar free chewing gum, jelly and pudding, coffee mix powder, biscuits, cookies, and cereal bars), resulting in an estimated daily mean intake of 1.62 g D-tagatose and 90th percentile daily intake of 3.89 g, is safe and GRAS for the general population.

This determination and notification are in compliance with proposed Sec. 170.36 of Part 21 of the Code of Federal Regulations (21 CFR section 170.36) as published in the Federal Register, Vol. 62, No. 74, FR 18937, April 17, 1997.

000003

Notifier's name and Address

CJ Cheiljedang, Inc.

Attention: Daniel Oh

Address: Namdaemunro 5-ga, Jung-gu, Seoul, Korea

Phone number: +82-2-726-8317

Fax number: +82-2-726-8319

E mail address: gethero@cj.net

Name of GRAS substance

D-Tagatose (Common or trade name: Tagatose or pseudo-fructose)

Product description

D-Tagatose is a ketohexose, an epimer of D-fructose isomerized at C-4. D-Tagatose differs from fructose only in the positioning of the hydroxyl group on the fourth carbon. D-Tagatose is 92% as sweet as sucrose. Functional uses of D-tagatose as food additives are as a sweetener, texturizer, stabilizer, humectant, and formulation aid. D-Tagatose provides several health benefits to consumers: 1) it does not promote tooth decay, 2) it provides approximately 1.5 kcal/g to the diet, and 3) it attenuates a glycemic response. D-Tagatose has a history of use in foods with no reported adverse effects.

Applicable conditions of use of the notified substance

D-Tagatose is expected to be used in ready-to-eat breakfast cereals, diet soft drinks, non-diet soft drinks, confectionery, formula diets for meal replacement, meal replacement drink mix (powder), hard and soft confectioneries, cake, pie, cake mix powder, frostings, ice cream and frozen yogurt, yogurt, frostings, chewing gum (sugar free), jelly and pudding, coffee mix powder, biscuits, cookies, and cereal bars. The proposed use levels of D-tagatose are presented in Table 1.

Table 1. Proposed food application of D-tagatose and maximum levels of use

Food category	Maximum level
Ready-to-eat breakfast cereals	10 g/30 g
Diet soft drinks	5 g/240 ml
Non-diet soft drinks	7.5 g/240 ml
Confectionery	7.5 g/30 g
Formula diets for meal replacement	7.5 g/240 ml
Meal replacement drink mix (powder)	2 g/6 g
Cake, pie,	10 g/100 g
Cake mix powder	15 g/100 g
Frostings	15 g/100 g
Ice cream and frozen yogurt	7.5 g/100 g
Yogurt	7.5 g/100 g
Chewing gum, sugar free	60%
Jelly and pudding	7.5 g/30 g
Coffee mix powder	7.5 g/20 g
Biscuits	10 g/100 g
Cookies	10 g/100 g
Cereal bars	10 g/40 g

Exposure estimates

If 10% of the products are used at the maximum levels, the mean intakes including D-tagatose from all GRAS proposed use categories by users of one or more foods is 1.62 g/d for the American population aged 1 and above. The 90th percentile intakes including D-tagatose from all GRAS proposed use categories by users of one or more foods are 3.89 g/d for the population combining males and females, 4.49 g/d for males and 3.24 g/d for females. These levels correspond to 69.5, 77.3, and 62.0 mg/kg BW/d for the entire population, males, and females aged 1 and over. These levels are much lower than the no-observed-adverse-effect level (NOAEL) values (2,300-6,700 mg/kg BW/d) that have been found from sub-chronic and chronic toxicity studies in rats. Also, these estimated daily exposure levels are far below the NOAEL value of 45 g/d that has been found from human clinical studies (Boesch et al., 2001; WHO, 2004).

Basis of GRAS determination

Through scientific procedures.

Review and copying statement

The data and information that serve as the basis for this GRAS determination will be sent to the FDA upon request, or are available for the FDA's review and copying at reasonable times at the office of CJ Cheiljedang, Inc. or Nutrasource, Inc.

We enclose an original and two copies of this notification for your review. If you have any questions, please contact me.

Sincerely,

(b) (6)

Susan Cho, Ph.D.
Chief Science Officer
NutraSource, Inc.
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Clarksville, MD 21029
Phone: 410-531-3336 (O) or 301-875-6454 (C)
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Conclusion of the expert panel:

Generally recognized as safe (GRAS) determination for the addition of D-tagatose to foods

August 2010

CONCLUSION

We, the undersigned expert panel members, Susan Cho, Ph.D., George Fahey, Ph.D., and James Anderson, M.D., have individually and collectively critically evaluated the materials summarized in the D-tagatose GRAS report and conclude that D-tagatose is safe and GRAS for its intended use in foods.

There is broad-based and widely disseminated knowledge concerning the chemistry of D-tagatose, a monosaccharide. D-Tagatose proposed for use in this food product is well characterized and free from chemical and microbial contamination. D-Tagatose will be used as an ingredient in foods and beverages.

Intended food applications include ready-to-eat breakfast cereals, diet soft drinks, non-diet soft drinks, confectionary, formula diets for meal replacement, cake, pie, cake mix powder, frostings, ice cream and frozen yogurt, yogurt, chewing gum (sugar free), jelly and pudding, coffee mix powder, biscuits, cookies, and cereal bars.

Assuming that 10% of the product will be used at the maximum levels under the intended use, the median intake including D-tagatose from the intended use by users of one or more foods is 1.62 g/d for the American population aged 1 and above. The 90th percentile intakes including D-tagatose from the intended use at the maximum levels by users of one or more foods are 3.89 g/d for the population combining males and females, 4.49 g/d for males and 3.24 g/d for females. These levels correspond to 69.5, 77.3, and 62.0 mg/kg body weight (BW)/d for the entire population, males, and females aged 1 and over. These levels are much lower than the no-observed-adverse-effect level (NOAEL) values (2,300-6,700 mg/kg BW/d) that have been found from sub-chronic and chronic toxicity studies in rats. Also, these estimated daily exposure levels are far below the NOAEL value of 45 g/d that has been found from human clinical studies.

Currently, D-tagatose is already listed as a GRAS ingredient. FDA had no question on Arla's GRAS notice on D-tagatose (GRN000078). The US FDA has allowed a health claim for D-tagatose and the risk reduction of dental caries in 2003. D-Tagatose has been formally approved as a 'novel food ingredient' in the European Union (EU) without any restriction on usages. The joint FAO/WHO Expert Committee on Food Additives (JECFA) concluded that D-tagatose was not genotoxic, embryotoxic or teratogenic, and identified a NOAEL of 0.75 g/kg BW/d (45 g D-tagatose/60 kg BW) in humans.

D-Tagatose has a long history of safe use as a component in natural foods. There are no indications of significant adverse effects related to D-tagatose in the publicly available literature. The proposed food use results in exposure at levels significantly below those associated with any adverse effects. Therefore, not only is the proposed use of D-tagatose safe within the terms of the Federal Food, Drug, and Cosmetic Act (meeting the standard of reasonable certainty of no harm),

but because of this consensus among experts, it is also *Generally Recognized as Safe* (GRAS) according to Title 21 Code of Federal Regulations (21 CFR).

Susan Cho, Ph.D.
President, NutraSource, Inc., Clarksville, MD 21029

(b) (6)
Signature: _____ Date: 8/12/10

George C. Fahey, Jr., Ph.D.
Professor, University of Illinois, Urbana, IL 61801

(b) (6)
Signature: _____ Date: 8/10/10

James Anderson, M.D.
Professor Emeritus, University of Kentucky, Lexington, KY 40502

(b) (6)
Signature: _____ Date: 8/11/10

Identity of substance

A. Common or trade name: D-Tagatose

B. Standards of identity: We note that an ingredient that is lawfully added to food products may be used in a standardized food only if it is permitted by the applicable standard of identity that is located in Title 21 of the Code of Federal Regulations.

C. Background:

D-Tagatose is a monosaccharide, an epimer of D-fructose isomerized at C-4. D-Tagatose differs from fructose only in the positioning of the hydroxyl group on the fourth carbon (Levin et al., 1995). D-Tagatose is 92% as sweet as sucrose and, therefore, contributes a similar amount of bulk to foods (Bertelsen et al., 1999; Levin et al., 1995). But it has less than half the calories of sucrose. It is odorless, white or almost white, non-hygroscopic. D-Tagatose is naturally present in human milk, sterilized (0.2-0.3%) and powdered cow's milk (0.01-0.1%), yogurts, cheeses (up to 0.01%), and some fruits (Levin et al., 1995).

Functional uses of D-tagatose as food additives are as a sweetener, texturizer, stabilizer, humectant, and formulation aid (FAO, 2004). D-Tagatose provides several health benefits to consumers: 1) it does not promote tooth decay (FDA, 2003a; Levin et al., 1995), 2) it provides approximately 1.5 kcal/g to the diet (Levin, 2002; Livesey and Brown, 1996), and 3) it attenuates a glycemic response (Donner et al., 1999). D-Tagatose has a history of use in foods with no reported adverse effects (Lu et al., 2008).

D-Tagatose is stable in the foods to which it is added during the processing and storage of food. However, D-tagatose may undergo Maillard reactions as a reducing sugar (FAO, 2004).

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D. Physico-Chemical properties of D-tagatose:

Synonym: D-lyxo-hexulose, α -D-tagatose, or pseudo-fructose

CAS registration number: 87-81-0

Empirical formula: $C_6H_{12}O_6$

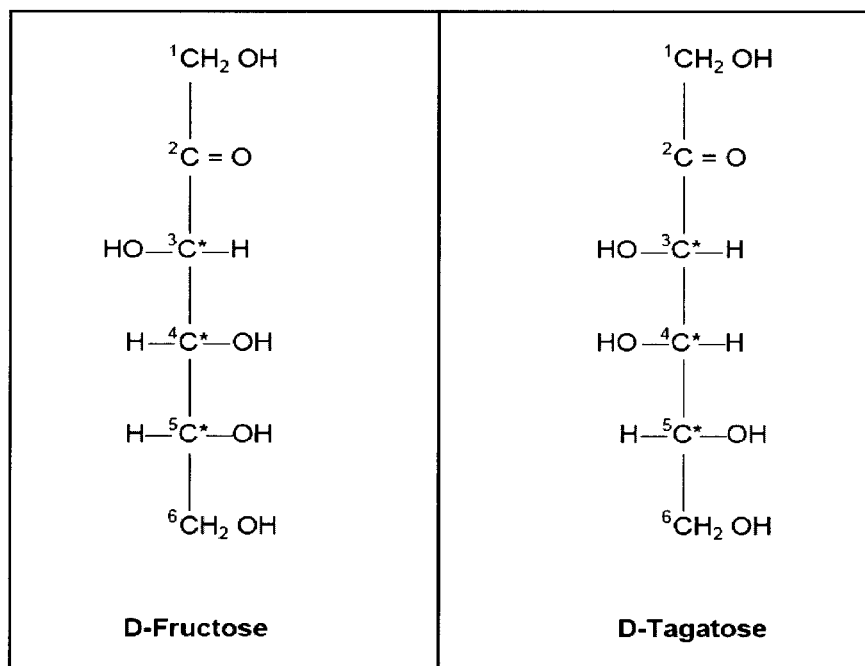
Molecular weight: 180.16 daltons

Structural formula: epimer of D-fructose

Solubility in water (g/100 ml at 20 °C): 160

Solubility in ethanol (g/100 ml at 22 °C): 0.02

Melting point (°C): 133 ~ 137



E. Manufacturing process:

The method of manufacture uses processes common to other food ingredients, such as high fructose corn syrup. D-Tagatose is produced from lactose by a two-step process involving acid hydrolysis of lactose to D-galactose, followed by enzymatic isomerization of D-galactose to D-tagatose (immobilized L-arabinose isomerase preparation). The final product is a highly purified crystal product.

- 1) The powder form of lactose is dissolved in clean water (20% solid concentration) in a reception tank.
- 2) 0.1% (w/w) of sulfuric acid is added and incubated at 140 °C for 1 h to hydrolyze lactose into D-glucose and D-galactose.
- 3) 0.1% of calcium carbonate (normally final pH is 5.5 - 7.0) is added to the hydrolyzed syrup to remove the precipitants, especially sulfate residues.
- 4) The neutralized syrup is mixed with manganese chloride (5 mM; 75 °C) and then subjected to an immobilized enzyme system (calcium alginate gel bead with *Corynebacterium glutamicum* (non-viable cell) harboring L-arabinose isomerase originated from *Thermotoga neapolitana*) at 75 °C for 4-8 h to convert D-galactose to D-tagatose.
- 5) The D-tagatose solution is mixed with 1 - 2 % of active carbon for 30 min in a stirred tank for de-colorization. The liquid undergoes pressure filtration *via* a Celite filter to clarify it (60 - 70 °C, pH > 4.5).
- 6) The decolorized syrup then is cooled to ≤ 40 °C, and then processed through an ion exchange process (i.e. cation column with strongly acidic cationic exchange resin; anion column with intermediate basic anion exchange resin; and a mixed bed column that has a combination of both strongly acid and strongly basic resins) to remove any impurities (e.g., calcium, manganese, chloride, and other ionic components including amino acids, peptides, and proteins). The exchange beds are monitored for pH and color every 8 h and the real-time conductivity is monitored automatically.
- 7) Following ion exchange purification, the D-tagatose solution is concentrated using an evaporator to produce a syrup (syrup density of 60° Brix (Bx)).

- 8) This concentrated syrup is pumped into a separation chromatography system to separate D-tagatose from other sugars (glucose, galactose, and unhydrolyzed lactose). This separation process dilutes the D-tagatose solution to a density of 10 - 15 °Bx.
- 9) Using an evaporator, the solution is concentrated to the final density of 75 - 80 °Bx.
- 10) The final concentrated product is pumped into a continuous crystallizer (30 h of retention time).
- 11) The crystalline D-tagatose is separated by basket centrifugation for 15 min, is washed by spraying distilled water, and is finally dried in a drum dryer.

Quality assurance procedure

Process tanks and lines are cleaned with sodium hydroxide and hydrogen peroxide following standard procedures common to the dairy industry. All ion exchange resins used for chromatographic purification of the D-galactose intermediate and D-tagatose, and for demineralization, comply with 21 C.F.R. § 173.25. Celite is cleared under 27 CFR § 24.243 (Filtering aids). Similar uses of activated carbon are considered GRAS for purification and clarification of wine as per 27 CFR §24.246. All processing aids used in the manufacturing process are considered safe and suitable.

The immobilized enzyme preparation is sterilized every three days with solution of food grade acetic acid, potassium sorbate, and sodium benzoate. The materials from enzyme sources are not included in the final product (rt-PCR and ELISA methods are used for verification).

F. Specifications:

Table 1 lists specifications of D-tagatose.

Table 1. Specifications of D-tagatose

Parameter	Specification
D-Tagatose	>98.5% (wt/wt)
D-Galactose and other sugars	≤ 1% (wt/wt)
Moisture	≤0.5 % (wt/wt)
Ash	≤ 0.1% (wt/wt)
Total plate count	<10,000 CFU/g
Coliforms	negative
<i>Staphylococcus aureus</i>	negative
Hg	<0.1 ppm
Cd	<0.1 ppm
Lead	<0.5 ppm
As	<1.0 ppm
Physical Appearance	White crystal

II. Natural occurrence and exposure to D-tagatose

A. Food sources of D-tagatose

D-Tagatose is found in low amounts in naturally occurring foods, such as sterilized milk products (0.2 - 0.3%), yogurt, milk-based infant formula, and hot cocoa (Levin et al., 1995; Troyano et al., 1991, 1996). It is also found in higher amounts (1%) in lactulose syrups (Zehner, 1994).

B. Intended use

D-Tagatose is intended to be used as a food ingredient. Intended food uses and use levels are summarized in Table 2. Considering its technological properties (such as function as a sweetener, humectant, flavor enhancer) and nutritional benefits (such as glycemic control), D-

tagatose is expected to be used in ready-to eat breakfast cereals, diet soft drinks, non-diet soft drinks, confectionery, formula diets for meal replacement, meal replacement drink mix (powder), cake, pie, cake mix powder, frostings, ice cream and frozen yogurt, yogurt, chewing gum (sugar free), jelly and pudding, coffee mix powder, biscuits, cookies, and cereal bars. The proposed use levels of D-tagatose are presented in Table 2.

Table 2. Proposed food application of D-tagatose and maximum levels of use

Food category	Maximum level
Ready-to-eat breakfast cereals	10 g/30 g
Diet soft drinks	5 g/240 ml
Non-diet soft drinks	7.5 g/240 ml
Confectionery	7.5 g/30 g
Formula diets for meal replacement	7.5 g/240 ml
Meal replacement drink mix (powder)	2 g/6 g
Cake, pie,	10 g/100 g
Cake mix powder	15 g/100 g
Frostings	15 g/100 g
Ice cream and frozen yogurt	7.5 g/100 g
Yogurt	7.5 g/100 g
Chewing gum, sugar free	60%
Jelly and pudding	7.5 g/30 g
Coffee mix powder	7.5 g/20 g
Biscuits	10 g/100 g
Cookies	10 g/100 g
Cereal bars	10 g/40 g

C. Current consumer intake levels

Since the D-tagatose level in each food is not listed in the USDA food composition tables and the National Health and Nutrition Examination Survey (NHANES) databases, the current exposure levels from food sources were not estimated. However, Zehner (1994) estimated that the exposure (mostly from lactulose over the counter syrups) before the intended use was 0.2 to 0.3 g/d.

D. Exposure estimates under the intended use

Using food intake data reported in the 2003-2006 NHANES, exposure levels to D-tagatose that will result from the intended uses were estimated (Table 3). The most recent NHANES (2003-2006) compiled by the National Center for Health Statistics and the Nutrition Coordinating Center was used to calculate exposure estimates. The NHANES was conducted between 2003-2006 with non-institutionalized individuals in the U.S. The NHANES provides the most current food consumption data available for the American population. The food and dietary supplement record for each individual includes the gram weight and nutrient data for all foods consumed during the day of the recall. All estimates were generated with USDA sampling weights to adjust for differences in representation of subpopulations. For this study 1 g is considered equivalent to 1 ml for soft drinks and formula diets for meal replacement. The NHANES does not include consumption levels of chewing gums. Thus, marketing survey data were used in exposure estimates: Average Americans eat 0.815 kg of gums/y (or average daily consumption of 2.29 g/person) and approximately 40% of chewing gums are sugar free. SUDAAN v10.0 with day 1 dietary weights are used to calculate mean, median, 90th percentile, and standard errors (SE) for D-tagatose exposure.

The intended use model is based on the assumption that 10% of the products in each category will be under the intended use. It is not possible to use all the foods under the intended use. Also wastage and other losses should be considered. Assuming that 10% of the product will be used at the maximum levels under the intended use, the mean intakes including D-tagatose from the intended use by users of one or more foods is 1.62 g/d for the American population aged 1 and above. The 90th percentile intakes including D-tagatose from the intended use by users of one or more foods are 3.89 g/d for the population combining males and females, 4.49

g/d for males and 3.24 g/d for females. These levels correspond to 69.5, 77.3, and 62.0 mg/kg BW/d for the entire population, males, and females aged 1 and over (Table 3-2). These levels are much lower than the no-observed-adverse-effect level (NOAEL) values (2,300-6,700 mg/kg BW/d) that have been found from sub-chronic and chronic toxicity studies in rats. Also, these estimated daily exposure levels are far below the NOAEL value of 45 g/d (or 15g three times a day) that has been found from human clinical studies (Boesch et al., 2001; Donner, 2006; WHO, 2004).

Table 3-1. Daily exposure estimate of D-tagatose under the intended use, g/day

Age	Gender	N	Exposure at Maximum Levels					
			Mean	SE	Med.	SE	P90	SE
1+ Y	All	17439	1.62	0.02	1.20	0.00	3.89	0.06
1+ Y	Male	8504	1.92	0.04	1.30	0.05	4.49	0.08
1+ Y	Female	8935	1.32	0.02	1.04	0.03	3.24	0.06

SE=standard error; Med.=Median; P90=90th percentile

Table 3-2. Daily exposure estimate of D-tagatose under the intended use, mg/kg BW/d

Age	Gender	N	Exposure at Maximum Levels					
			Mean	SE	Med.	SE	P90	SE
1+ Y	All	17274	28.21	0.39	17.58	0.36	69.52	1.29
1+ Y	Male	8431	31.65	0.62	20.17	0.56	77.29	2.09
1+ Y	Female	8843	24.91	0.48	15.81	0.40	61.98	1.33

SE=standard error; Med.=Median; P90=90th percentile

From a marketing perspective, an assumption that 10% of the product will be used at the maximum levels for each food category is a highly optimistic projection. The global functional foods market is expected to grow through 2010 (Market Research.com, 2004). After 2010, the functional foods market size is expected to comprise approximately 5% of total food expenditures in the developed world. Assuming that 10% of the product will be used at the maximum levels under the intended use, annual D-tagatose consumption of 177,390 tons is

expected in the U.S. (based on the estimated mean daily intake level of 1.62 g/person/d and the US population of 300,000,000 persons). This projection might be higher than actual consumption levels of D-tagatose in the U.S. The US consumption of all types of sugar alcohols (sorbitol, erythritol, maltitol and xylitol) was estimated at 376,640 tons (or 3.43 g/person/d) in 2002, of which sorbitol made up the largest percentage, with more than 54% of the total sugar alcohol production (Food Navigator, 2003). Actual US consumption of D-tagatose was estimated to be lower than the figure for any single sugar alcohol.

III. Basis for GRAS determination

A. Current regulatory status

D-Tagatose is classified as GRAS (generally recognized as safe) by the United States Food and Drug Administration (GRN 000078; FDA, 2003b). The US FDA has allowed a health claim for tagatose and the risk reduction of dental caries in 2003 (FDA, 2003a). D-Tagatose has been formally approved as a 'novel food ingredient' in the European Union (EU) without any restriction on usages (FSA, 2005). It also has been approved for use in other countries including Australia, New Zealand, South Korea, Brazil, and South Africa (FSA, 2005; FSANZ, 2004; Kroger et al., 2006).

The joint FAO/WHO Expert Committee on Food Additives (JECFA) evaluated D-tagatose at its 55th, 57th, 61st and 63rd meetings (FAO, 2001, 2004, 2005; JECFA, 2001; WHO, 2001, 2004, 2006). At the 55th, 57th, and 61st meetings of JECFA, the Committee concluded that D-tagatose was not genotoxic, embryotoxic, or teratogenic. At the 57th meeting, the JECFA identified a NOAEL of 0.75 g/kg BW/d (45 g tagatose/60 kg BW) and an adequate daily intake (ADI) of 0–80 mg/kg BW for D-tagatose. An ADI was determined based on the NOEL and a

safety factor of 10 (FAO, 2004). However, at the 61st meeting in June, 2004, JECFA stated that there is no need to limit the allowed daily intake of D-tagatose (WHO, 2006).

B. Intended technical effects

D-Tagatose has been used as a food ingredient. D-Tagatose can serve as a bulk sweetener, humectant, texturizer, and stabilizer. Also, D-tagatose can be used as a non-cariogenic ingredient for glycemic control (Lu et al., 2008).

B. Review of safety data

The predominant effects associated with excessive consumption were related to gastrointestinal effects, liver enlargement, and elevated uric acid concentration (INCHEM, 2006). The panel recognizes that D-tagatose has been subjected to thorough toxicological testing and supports the conclusion of JECFA of FAO/WHO (WHO 2004, 2006) that D-tagatose is safe at the daily doses up to 45 g.

1. Metabolism of D-tagatose

Feeding studies of D-tagatose using pigs and rats showed that approximately 25% of the amount of D-tagatose ingested is passively absorbed into the blood stream (Bar, 2004; FSANZ, 2004; Johansen and Jensen, 1997; Saunders et al., 1999a). A metabolism study in rats has shown that radio-labeled carbon (^{14}C) of orally administered ^{14}C -D-tagatose is excreted in the urine (5–6%), breath (49–68%) and feces (11–29%) (Levin et al., 1995). In pigs, the digestibility of D-tagatose was estimated to be $25.8 \pm 5.6\%$ in the distal third of the small intestine. In this study, 8 pigs were fed a low fiber diet comprising 15% sucrose (control group). Another eight pigs were

fed a similar diet except that 10% sucrose in diet was replaced by D-tagatose (test group) for 18 d (Laerke and Jensen, 1999).

In humans, the absorption of D-tagatose cannot be measured directly. A human study with 6 ileostomy subjects suggests that the apparent absorption of 15 g D-tagatose/d was 81% (Normen et al., 2001). Approximately 19% (range: 12-31%) ingested D-tagatose was recovered from the 24-h ileal effluents. Of the total amount of D-tagatose excreted [2.8 g (1.7-4.4 g)], 60% (8-88%) was excreted within 3 h. Between 3 and 5 h, 32% (11-82%) was excreted.

In a pig study of Laerke and Jensen (1999), no D-tagatose was recovered from feces and D-tagatose had effects on reducing the luminal pH and increasing the concentrations of propionate and butyrate in the cecum and colon. These results indicate that the D-tagatose which escaped the upper gastrointestinal tract was almost completely fermented in the cecum and proximal colon and only small amounts reached the more distal parts of the colon. Fermentation in the lower gut is not unique to D-tagatose. Carbohydrates that escape the small intestine are fermented by intestinal microflora to produce short chain fatty acids (SCFAs). When 30 g D-tagatose was administered in 8 healthy subjects, there was a significant increase in 24-h H₂ production (35%), indicating a substantial fermentation of malabsorbed D-tagatose in the lower gut (Breumann et al., 1998).

The liver appears to be the primary site of tissue uptake, with little D-tagatose reaching the systemic circulation. When 30 -75 g D-tagatose were administered to healthy subjects, serum concentrations peaked after 50-90 min, with a range of 0.05 to 0.28 mmol/l (Buemann et al., 2000a; Donner et al., 1999) and D-tagatose was not detected in the serum after 7 h (Buemann et al., 2000a). The caloric value of D-tagatose was estimated to be approximately 1.5 kcal/g (Livesey and Brown, 1996).

The 61st JECFA considered the risk to individuals with hereditary fructose intolerance (WHO, 2006). Treatment requires almost complete exclusion of sucrose, fructose, sorbitol, and fiber ingredients with fructose backbone, such as inulin and fructooligosaccharides. The 2006 WHO report states that “---the committee noted that the absorption of D-tagatose by humans is not expected to exceed 20% of the administered dose. However, the rate of gluconeogenesis from D-tagatose is slower than that from fructose. Thus the Committee could not discount the possibility that, in individuals with hereditary fructose intolerance, tissue concentration of D-tagatose could be elevated or prolonged with those of fructose, leading to adverse reactions.”

2. Safety/Toxicity of D-tagatose—Preclinical studies (mostly adopted from GRN 000078; WHO 2004, 2006 as well as original research articles)

The panel agrees with the previous reviews (GRN 000078 and WHO) which state that tagatose was safe at the 5% level in the diet (Kruger et al., 1999 a,b,c; Lina and de Bie, 2000, Lina and Bar, 2003; Trimmer, 1989). Table 4 summarizes acute, subacute, subchronic, and chronic toxicity studies which focused on hepatic effects of D-tagatose such as increased liver weight and hypertrophy. As shown in Table 4, Sprague-Dawley rats were more sensitive to these hepatic effects than Wistar rats: No abnormalities were observed in Wistar rats fed 10% D-tagatose in diet (Appel, 2002; Lina and de Bie, 2000) although Sprague-Dawley rats had the NOAEL of 5% in diet (Bär, 1999; Kruger et al., 1999a). The WHO (2006) referenced a 2-year chronic toxicity/carcinogenicity study in which 10% D-tagatose in diet (or 4-5 g/kg BW/d) produced liver glycogen deposition and hypertrophy with no histopathological changes in Wistar rats (Lina and Bär, 2003; original research data are not available). The NOAEL of 5% in diet was determined based on the studies using Sprague-Dawley rats.

Fructose and D-tagatose have a similar chemical structure and share a common metabolic pathway following an initial phosphorylation step (Bär, 2000). When D-tagatose enters the liver, it is phosphorylated by ketohexokinase to tagatose-1-phosphate (tagatose-1-P) which is further cleaved by aldolase B to glyceraldehyde (GA) and dihydroxyacetone phosphate (DHAP; Espinosa and Fogelfeld, 2010). The latter step occurs at a slower rate than the phosphorylation step, which leads to a series of metabolic pathways including accumulation of tagatose-1-P, stimulation of glucokinase, an increase in conversion of glucose to glucose-6-P, and, finally, hepatic glycogen synthesis. Also, tagatose-1-P inhibits glycogen phosphorylase which results in a decrease of conversion of glycogen into glucose-1-P. Thus, it has been postulated that D-tagatose has both effects of stimulating glycogen synthesis and decreasing glycogen breakdown. However, it should be noted that all of these studies have been conducted either in *in vitro* or using animal models and that no human studies have confirmed this mechanism of D-tagatose action (Espinosa and Fogelfeld, 2010).

It is noteworthy that liver enlargement is not unique to D-tagatose consumption. D-Fructose and L-sorbose, a stereoisomer of fructose, caused increased liver weights and increased accumulation of glycogen in rats after administration of large amounts of D-fructose (15-20% in the diet; Murakami et al., 1997) and L-sorbose (10-20% in the diet; Fruruse et al., 1989).

Table 4. Acute, subacute, subchronic, and chronic toxicity studies of D-tagatose in animals

Species (No. of animals/ group)	Route	D- Tagatose, conc.	Duration	Result	NOAEL	Reference
Rat (5M, 5F) and mice (5M)	Oral, gavage	10 g/kg BW	Single dose	No treatment effects.	10 g/kg BW	Trimmer, 1989
Rat, S-D (20M, 20F)	Feed	5, 10, 15, and 20% in diet	90 d	No abnormalities at 5% (or 2,300-6,700 mg/kg BW/d); Increased rel. liver wt. at 10, 15, 20%; Some hypertrophy of hepatocytes at 15 and 20%.	5% in diet	Kruger et al., 1999a
Rat, S-D (20M)	Feed	20%	2 and 4 wk	Increased levels of liver wt and glycogen; Slightly increased ALAT and ASAT.		Bär, 1999
Rat, S-D (20M)	Feed	5,10, and 20% in diet	29-31 d	10-20% - Dose-dependent increase of liver wt and glycogen. No other morphological changes of liver tissue; 20%-Slight increase in ALAT and ASAT	5% in diet	Bär, 1999
Rat, S-D (20M)	Feed	5% in diet	4 wk	Fasted rats - no abnormalities; Non-fasted rats-increased liver wt; Overall, no hepatocellular growth at 5% dietary level (or 2600-2800 mg/kg BW/d).	5% in diet	Bär, 1999
Rat, Wistar (60F)	Feed	5-10% in diet	Up to 6 mo	No abnormalities.	10% in diet	Lina and de Bie, 2000
Rat, Wistar (60F)	Feed	5-10% in diet	2 wk	No abnormalities.	10% in diet	Lina and de Bie, 2000
Rat, Wistar	Feed	0, 2.5, 5, 10% tag, 20% fru, 10% tag + 10% fru in diet	24 mo	No morphological changes at up to 10%; Enlarged liver in 10% tag (M), 20% fru (M), 10% tag + fru (M+F); Increased nephro-calcinosis in females of all tag dose groups and in 10% tag (M) and 10% + 10% fru (M); Increased incidence of adrenomedullary proliferative disease in 2.5% tag (M), 5% tag (M+F), 10% (M+F) and 10% +10% fru (M+F).	10% in diet	Lina and Bär, 2003
6 strains of rats	Feed	20% in diet (or 13 g /kg BW/d)	4 wk	Increased liver weight.		Appel, 2002
Pig (2)	Feed	Up to 20% in diet (or 5 g /kg BW/d)	33 d	No abnormalities.	5 g/kg BW/d	Mann, 1997

Tag = tagatose; fru = fructose; M = males; F = females; S-D = Sprague-Dawley;
Six strains = Lewis, Fischer, Brown Norway, Lister Hooded, S-D, and Wistar

The panel also agrees with the previous reviews (GRN 000078; WHO, 2004, 2006) which address that D-tagatose did not show genotoxicity, developmental toxicity, or reproductive toxicity. The WHO report (2006) states that “At its 55th meeting, the Committee concluded that D-tagatose was not genotoxic, embryotoxic or teratogenic.” Table 5 summarizes a developmental and reproductive toxicity study. Reproductive performance was not affected by the treatment of up to 20 g/kg BW/d (Table 5; Kruger et al., 1999b). Maternal liver weight increased in the 12 and 20 g/kg BW/d groups with no morphological changes in liver. No other adverse effects were noted.

Table 5. A developmental and reproductive toxicity study of D-tagatose

Species	Route	Tagatose, conc.	Duration	Result	NOEL	Reference
Rat, S-D (24M / group)	Feed	0, 4, 12, 20 g/kg BW/d	(day 6-15 of gestation)	Maternal liver weight increased in 12 and 20 g/kg BW/d group; No morphological changes in liver; No other adverse effects.	20 g /kg BW/d	Kruger et al., 1999b

D-Tagatose at concentrations up to 5 mg/plate or 5 mg/ml was not found to be genotoxic when the genotoxicity was examined in five standard assays: the Ames *Salmonella typhimurium* reverse mutation assay, the *Escherichia coli*/mammalian microsome assay, a chromosomal aberration assay in Chinese hamster ovary cells, a mouse lymphoma forward mutation assay, and an *in vivo* mouse micronucleus assay (Table 6; Kruger et al., 1999c).

Table 6. Genotoxicity studies showing no adverse effects of D-tagatose

Test	Test system	Concentration of D-tagatose	Reference
Bacterial gene mutation, with and without rat liver S9 metabolic activation	<i>S.typhimurium</i> (TA 1535, TA 1537, TA1538, TA98, TA100); <i>E.coli</i> (WP2uvrA)	100-5,000 ug/plate	Kruger et al., 1999c
Chromosomal aberration, with and without rat liver S9 metabolic activation	Chinese hamster ovary cells	1,250-5,000 ug/ml	Kruger et al., 1999c
Micronucleus formation, up to 72 h after dosing	CD-1 mouse bone marrow	1,250-5,000 mg/kg BW	Kruger et al., 1999c

3. Safety of D-tagatose - Human clinical studies

The panel supports the WHO conclusion that a daily dose of 45 g D-tagatose (or 0.75 g/kg BW/d) is considered as a NOAEL in humans. Table 7 summarizes the results of human clinical trials in which healthy and/or diabetic subjects were administered daily D-tagatose doses of 15-75 g for up to 1 y (Boesch et al., 2001; Buemann et al., 1999a,b,c, 2000b; Donner et al., 1999; Donner, 2006; Lee and Storey, 1999; Saunders et al., 1999b).

Several clinical studies with D-tagatose have evaluated potential uricemic effects. The presence of chronically elevated plasma uric acid levels (i.e., hyperuricemia) is known to increase the risk of gout, a purine metabolism disorder. It is related to accumulation of ketohexose-1-phosphate which results in hepatocellular depletion of inorganic phosphate and the degradation of purine nucleotides (WHO, 2004). D-Fructose also increases plasma uric acid concentrations, but at a slower rate than D-tagatose. The ingestion of single high bolus doses of D-tagatose produced a mild, transient increase of plasma uric acid concentration in both healthy subjects and subjects with non-insulin dependent diabetes (NIDDM; Buemann et al., 2000b; Saunders et al., 1999b). However, in longer term studies, daily doses of up to 45 g (i.e., 15 t.i.d.) for 28 d - 1yr showed no physiologically significant effects of D-tagatose on fasting plasma uric

acid levels in both healthy and NIDDM subjects (Boesch et al., 2001; Saunders et al., 1999b). Any increases in plasma uric acid concentrations were within the normal range (Buemann et al., 2000b; Diamantis and Bär, 2001, 2002).

WHO noted that D-fructose increases uric acid production by accelerating the degradation of purine nucleotides, probably by hepatocellular depletion of inorganic phosphate resulting from accumulation of ketohexose-1-phosphate (WHO, 2004). The degradation of D-tagatose-1-phosphate is slower than that of D-fructose-1-phosphate, and therefore the hyperuricemic effect of D-tagatose may be greater than that of D-fructose; hyperuricemic individuals are therefore potentially vulnerable to the adverse effects of D-tagatose. However, there was no increase in serum concentrations of uric acid within 4 h of consumption of 15 g of D-tagatose by this vulnerable group and no effect would be observed in hyperuricaemic individuals after repeated consumption of 15 g of D-tagatose at subsequent meals (Diamantis and Bär, 2001, 2002, cited in the 2004 WHO Food Additive Series 52 report).

Table 7. Safety studies in humans

Subjects	Daily dose of tagatose	Duration	Results	NOEL	Reference
Effects on liver					
12 healthy males	45 g (15g tid)	28 d	No effect on liver volume or glycogen conc.	45 g/d	Boesch et al., 2001
Effects on plasma uric acid conc.					
12 healthy males	45 g (15g tid)	28 d	No effect on plasma uric acid conc.	45 g/d	Boesch et al., 2001
8 NIDDM	45 g (15g tid)	1 y	No effect on plasma uric acid conc. Decreased rise in postprandial glucose	45 g/d	Donner, 2006
8 healthy and 8 NIDDM	75 g (25g tid)	8 wk	A transient increase of plasma uric acid conc. after single doses of 75 g of D-tagatose in the tolerance test, but not after 8 wk.		Saunders et al., 1999b
8 healthy males	30 g	Single dose	Increased serum uric acid conc., which was within the normal range.	30 g/d	Buermann et al., 1999b
6 healthy males	30 g	Single dose	Increased serum uric acid conc., which was within the normal range.	30 g/d	Diamantis and Bär, 2001
12 hyper-uricaemic males	15 g	Single dose	Increased serum uric acid conc. by 2.5%.	15 g/d	Diamantis and Bär, 2002
Gastrointestinal effects					
33 healthy volunteers	29 g	Single dose	Increased GI symptoms on the 1st day, but no treatment effects on the 2nd day.	29 g/d	Buermann et al., 1999a
8 healthy and 8 NIDDM	75 g	Single dose	Increased GI symptoms.		Donner et al., 1999
Healthy volunteers	30 g	Single dose	Well tolerated.	30 g/d	Buermann, 1998
50 healthy adults	20 g	Single dose	Well tolerated.	30 g/d	Lee and Story, 1999
73 healthy males	29-30 g (single dose each day)	15 d	Nausea in 15.1% and diarrhea in 31.5% during screening. Light or moderate flatulence.	29-30 g/d	Buermann et al., 1999c
12 healthy males	45 g (15g tid)	4 wk	Well tolerated. Laxation effects noted for 7/12 subjects.	45 g/d	Boesch et al., 2001
8 NIDDM	45 g (15g tid)	1 y	No effect on plasma uric acid conc.; decreased rise in postprandial glucose; GI symptoms in all subjects	45 g/d	Donner, 2006
19 healthy males	29 g	Single dose	2 cases of moderate nausea, 1 case of strong flatulence	29 g	Beumann, 2000
8 healthy and 8 NIDDM	75 g (25 g tid)	8 wk	Flatulence in 7/8; diarrhea in 6/8 subjects		Saunders et al., 1999b

GI symptoms = diarrhea, nausea, and/or flatulence; tid= ter in die (Latin: three times a day)

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Gastrointestinal effects of D-tagatose are related to its incomplete absorption in the small intestine and are not unique to D-tagatose (Saunders et al., 1999b). Excessive consumption of sugar alcohols and dietary fibers also results in gastrointestinal symptoms, such as bloating, abdominal pain, and diarrhea (Grabitske and Slavin, 2009). It may be due to a combination of an osmotic effect in the small intestine of unabsorbed carbohydrates and fermentation in the large intestine (Grabitske and Slavin, 2009). Carbohydrates that escape the small intestine are fermented in the large intestine to produce SCFA and gases (IOM, 2002). On the other hand, such incomplete absorption of carbohydrates often provides several health benefits, such as prebiotic effects, improved gastrointestinal health (such as relieving constipation), and the risk reduction of cardiovascular diseases and diabetes (Cho and Finocchiaro, 2009; Cho and Samuel, 2009; IOM, 2001). In fact, other indigestible carbohydrates such as psyllium and inulin are commercially marketed as laxatives to promote intestinal regularity. For example, Metamucil[®] formulated with psyllium is marketed as a laxative by Proctor and Gamble; FiberChoice[®] containing inulin is marketed by GSK.

5. Perspective of WHO 2004 and 2006

Studies of D-tagatose administered to rats in the diet, reviewed previously by the WHO Committee (WHO 2004, 2006), focused on the hepatic effects of D-tagatose, in particular, increased liver weight and hypertrophy. These studies indicated that these effects were caused, at least in part, by glycogen accumulation, and that Sprague-Dawley rats were more sensitive to these effects than Wistar rats.

At its 57th meeting, the Committee identified a NOAEL for healthy individuals of 45 g of D-tagatose per day in three divided doses (WHO, 2004). In addition, the WHO noted the fact

that ingestion of 30 g or more of D-tagatose on a single occasion may cause gastrointestinal effects in humans (WHO, 2006).

The study on hyperuricemic individuals discussed at the meeting indicated that the NOAEL is also applicable to this vulnerable group. On the basis of the NOAEL of 0.75 g/kg BW per day, and a safety factor of 6 (to allow for inter-individual variation), the Committee allocated a temporary ADI for D-tagatose of 0–125mg/kg BW. The temporary ADI did not apply to individuals with hereditary fructose intolerance caused by deficiency in 1-phosphofructoaldolase (aldolase B) or fructose 1,6-diphosphatase.

The WHO (2006) states the following in its Food Additive Series 54: “At its sixty-first meeting, the Committee concluded that, pending provision of the results of histopathological examinations from a 2-year study in rats, the human data provided the most relevant basis for assessing the acceptable intake of D-tagatose. The histopathological data had now been provided and demonstrated that there was no toxicologically significant findings in rats given D-tagatose at levels of up to 10% in the diet for 2 years (equal to approximately 4 and 5 g/kg bw per day for males and females, respectively). On the basis of the data reviewed by the Committee at its sixty-first meeting and at its present meeting, and taking into account the fact that D-tagatose has physiological and toxicological properties similar to those of other carbohydrates of low digestibility, the Committee removed the temporary ADI and allocated an ADI 'not specified' for D-tagatose. --- The ADI 'not specified' does not apply to individuals with hereditary fructose intolerance arising from 1-phosphofructoaldolase (aldolase B) deficiency or fructose 1,6-diphosphatase deficiency.”

It is noteworthy that certain segments of the population are allergic/intolerant to wheat (gliadin), milk (lactose), peanuts, and egg protein. That does not mean that wheat, milk, peanut,

or eggs are not GRAS. The issue of hereditary fructose intolerance can be treated in a similar manner to allergies/intolerance to other food substances listed above.

Safety of *Corynebacterium glutamicum* harboring L-arabinose isomerase originated from *Thermotoga neapolitana*

The safety of L-arabinose isomerase originated from *Thermotoga neapolitana* has been tested in a 14 d mouse study (up to 2000 mg/kg BW/d), bacterial reverse mutation tests (*Salmonella typhimurium*, TA100, TA1535, TA98, and TA1537, and *Escherichia coli*, WP2 uvrA; up to 5,000 µg/plate), and in *in vitro* mammalian chromosomal aberration test (D-tagatose conc. up to 5 mg/ml). No adverse effects were noted at these levels (see appendix for details).

Safety of *Corynebacterium glutamicum*

Corynebacterium glutamicum has been known as an ideal organism for industrial production of functional food ingredients, such as xylitol and amino acids. It is due to its ease of gene and pathway manipulations, rapid growth, and ease of high-cell density cultivation in inexpensive growth media (Kim et al., 2010; Smid et al., 2005). The microorganism is generally recognized as safe. In 1957, Kinoshita et al. discovered *C. glutamicum* (initially named *Micrococcus glutamicus*) as a potent amino acid-producing microorganism (Tryfona and Bustard, 2005). Since that time, *C. glutamicum* has been widely used in the production of various amino acids (approximately 900,000 tons/y) including L-glutamic acid, L-lysine and DL-methionine (Tryfona and Bustard, 2005). Recently, *C. glutamicum* has been used for production of xylitol and tagatose (Kawaguchi et al., 2009; Kim et al., 2010) due to its stability under high osmotic pressure generated by a high concentration of galactose. FDA (2005) has approved Monsanto's

Biotechnology Notification File (BNF 000087), genetically modified maize containing the cordapA gene derived from Corynebacterium glutamicum.

4. Summary of safety studies

D-Tagatose, a stereoisomer of fructose, has been proposed for use as a food ingredient in the preparation of low- and reduced-calorie foods. Currently, trace levels of D-tagatose are consumed from the diet, as a result of its presence in sterilized milk products (formed from lactose by heat treatment). Additionally, consumption of lactulose syrups (OTC preparations) results in exposures of 0.2 to 0.3 g/d.

The panel supports the conclusions of the WHO reports (WHO, 2004, 2006) that D-tagatose was safe at the 5% dietary level (2.3 to 6.7 g/kg BW/d) in rats and at the daily doses up to 45 g (or 0.75 g/kg BW/d) in humans. Data supporting the safety of D-tagatose include several clinical trials in healthy and diabetic volunteers, animal toxicology studies, and genotoxicity studies (Boesch et al., 2001; Buemann et al., 1999a,b,c, 2000b; Donner et al., 1999; Donner, 2006; Kruger et al., 1999a,b,c; Lee and Storey, 1999; Saunders et al., 1999b; Trimmer, 1989). Although the mechanism for potential effects of D-tagatose on uric acid is conjectural, any possible effect of D-tagatose on uric acid, even of a mild nature, would occur only at exposures several fold in excess of the 90th percentile estimated daily intake. Although the mechanism for potential effects of D-tagatose on uric acid is conjectural, any possible effect of D-tagatose on uric acid, even of a mild nature, would occur only at exposures several fold in excess of the 90th percentile estimated daily intake.

Assuming that 10% of the product will be used at the maximum levels under the intended use, the mean intakes including D-tagatose from the intended use by users of one or more foods

is estimated at 1.62 g/d for the American population aged 1 and above. The 90th percentile intakes including D-tagatose from all the intended use at the maximum levels by users of one or more foods are 3.89 g/d for the population combining males and females, 4.49 g/d for males and 3.24 g/d for females (Table 3-1). These levels correspond to 69.5, 77.3, and 62.0 mg/kg BW/d for the population (combining males and females), males, and females aged 1 and over (Table 3-2). These levels are much lower than NOAEL values (2,300-6,700 mg/kg BW/d) that have been found from sub-chronic and chronic toxicity studies in rats and the NOAEL value of 750 mg/kg BW/d that has been found from human clinical studies.

IV. Conclusions

The information/data provided by CJ CheilJedang (specifications, manufacturing process, and intended use) in this report and supplemented by the publicly available literature/toxicity data on D-tagatose provide a sufficient basis for an assessment of the safety of D-tagatose for the proposed use as an ingredient in food, when prepared according to appropriate specifications and used according to GMP. Key findings are summarized here:

1. D-Tagatose is well characterized and free from chemical and microbial contamination.
2. D-Tagatose has a long history of use in foods in the U.S.
3. The safety and nutritional benefits of D-tagatose are well established by human clinical trials and animal studies of D-tagatose.
4. Intended use of D-tagatose as part of the proposed food use (ready-to-eat breakfast cereals, diet soft drinks, non-diet soft drinks, confectionary, formula diets for meal replacement, cakes, pies, cake mix powder, frostings, ice cream and frozen yogurt, yogurt, sugar free chewing gum, jelly and pudding, coffee mix powder, biscuits, cookies, and cereal bars, even at the 90th percentile, results in levels of exposure significantly below those associated with any adverse effects and provides a reasonable certainty of safety.

Therefore, not only is the proposed use of D-tagatose safe within the terms of the Federal Food, Drug, and Cosmetic Act (meeting the standard of reasonable certainty of no harm), but because of this consensus among experts, it is also *Generally Recognized as Safe* (GRAS).

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V. Discussion of Information Inconsistent with GRAS Determination

We are not aware of information that would be inconsistent with a finding that the proposed use of D-tagatose preparations in foods and beverages, meeting appropriate specifications and used according to GMP, is GRAS.

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FINAL REPORT

***In vitro* Mammalian Chromosome Aberration Test
of L-Arabinose Isomerase (EC 5.3.1.4) in
Cultured Chinese Hamster Lung (CHL) Cells**

Study Number: 10-VG-060

Sponsor: CJ Cheiljedang Food R&D

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Translation Verification Statement

This report is a translation of original Korean Final Report issued on Jun 07, 2010.

Hereby, I certify that the contents are accurately translated.

(b) (6)

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Jul 07, 2010

Date

Statement of Management

Title: *In vitro* Mammalian Chromosomal Aberration Test of L-Arabinose Isomerase (EC 5.3.1.4) in Cultured Chinese Hamster Lung (CHL) Cells

The above study was conducted in accordance with the following GLP's and Guidelines.

1. The Standards of Toxicity Study for Medicinal Products (Notification No. 2009-116, Korea Food and Drug Administration, Aug 24, 2009)
2. Guideline No. 15 '*In vitro* Chromosome Aberration Test' of Section 4 Health Effects, the National Institute of Environmental Research Notification No. 2009-57 'Good Laboratory Practice Standards and Test Guidelines' (Nov 24, 2009)
3. OECD Guideline for Testing of Chemicals, TG 473 (1997) '*In Vitro* Mammalian Chromosomal Aberration Test'
4. Good Laboratory Practice (Notification No. 2009-183, Korea Food and Drug Administration, Dec 22, 2009)
5. Good Laboratory Practice Standards, National Institute of Environmental Research Notification No. 2009-57 'Good Laboratory Practice Standards and Test Guidelines' (Nov 24, 2009)
6. OECD Principles of Good Laboratory Practice (1997)

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Quality Assurance Statement

Study No.: 10-VG-060

Title: *In vitro* Chromosomal Aberration Test of L-Arabinose Isomerase (EC 5.3.1.4) in Cultured Chinese Hamster Lung (CHL) Cells

Study period: Mar 29, 2010 – Jun 07, 2010

Sponsor: CJ Cheiljedang Food R&D

Items	Inspected on	Inspection results reported to Study Director on	Inspection results reported to Management on
Protocol	Mar 26, 2010	Mar 29, 2010	Mar 30, 2010
Storage of cells and culture initiation	Apr 06, 2010	Apr 06, 2010	Apr 08, 2010
Subculturing	Apr 06, 2010	Apr 06, 2010	Apr 08, 2010
Storage of test/reference article	Apr 08, 2010	Apr 08, 2010	Apr 13, 2010
Preparation of test/reference article	Apr 08, 2010	Apr 08, 2010	Apr 13, 2010
Status of cells	Apr 08, 2010	Apr 08, 2010	Apr 13, 2010
Identification of cultures	Apr 08, 2010	Apr 08, 2010	Apr 13, 2010
Chemical treatment	Apr 08, 2010	Apr 08, 2010	Apr 13, 2010
Preparation of slides	Apr 09, 2010	Apr 09, 2010	Apr 13, 2010
Slide reading	Apr 29, 2010	Apr 29, 2010	Apr 29, 2010
Raw data	May 13, 2010	May 17, 2010	May 20, 2010
Final report (draft)	May 13, 2010	May 17, 2010	May 20, 2010
Final report	Jun 07, 2010	-	-

Hereby, I do certify that the detailed methods, procedures, and observations in this report are accurately and completely described, and that the reported results accurately and completely reflect the raw data, and that this study was performed in conformity with the Korea Food and Drug Administration (KFDA) Notification No. 2009-116 'The Standards of Toxicity Study for Medicinal Products' (Aug. 24, 2009); Guideline No. 15 '*In vitro* Chromosome Aberration Test' of Section 4 Health Effects, the National Institute of Environmental Research Notification No. 2009-57 'Good Laboratory Practice Standards and Test Guidelines' (Nov 24, 2009); OECD Guideline for Testing of Chemicals, TG 473 (1997) '*In Vitro* Mammalian Chromosomal Aberration Test'; Good Laboratory Practice (Notification No. 2009-183, Korea Food and Drug Administration, Dec 22, 2009); Good Laboratory Practice Standards, National Institute of Environmental Research Notification No. 2009-57 'Good Laboratory Practice Standards and Test Guidelines' (Nov 24, 2009) and OECD Principles of Good Laboratory Practice (1997).

Jun 07, 2010

(Signature in the original report)

Moon-Soon Kim
Quality Assurance Manager,
Preclinical Research Center, ChemOn Inc.

Synopsis

Title *In vitro* Mammalian Chromosome Aberration Test of L-Arabinose Isomerase (EC 5.3.1.4)) in Cultured Chinese Hamster Lung (CHL) Cells

Objectives This study was carried out to evaluate the mutagenic potential of the test article, L-Arabinose Isomerase (EC 5.3.1.4), in terms of clastogenicity using cultured Chinese Hamster Lung (CHL) cells.

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Schedule Mar 29, 2010: Approval of protocol (Study initiation)
Apr 05, 2010: Set up cultures (Experimental initiation)
Apr 08, 2010: Chemical treatment
Apr 09, 2010: Preparations of slides
May 05, 2010: Completion of slides reading (Experiment completion)
May 19, 2010: Submission of Draft report
Jun 07, 2010: Submission of Final report (Study completion)

Archives The protocol and amendments, final report, raw data, specimens, sample of test article, and relevant documents produced during the present study period will be retained and stored in the Archives of Preclinical Research Center, ChemOn Inc. for three years after the completion of the study. Further storage of above materials shall be consulted with the sponsor.

Contributing Scientists

DIVISIONS	NAME	DUTIES
Cell culture and Testing	Seong-Sook Kim, M.S.	Study Director
Storage/Preparation of the Test article	Hyun-Suk Heo	Chief of Preparation and Storage of Test Articles
Slide preparation and reading	Seong-Sook Kim, M.S.	Study Director
Statistics	Min-Hang Lee, M.S.	Chief of Statistics
Peer Review	Hyun-Kul Lee, Ph.D. Toxicologist	Peer review of study protocol and report
Archives	Jin-Young Lee	Chief of Archives

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1. Summary

The objective of this study was to evaluate the mutagenic potential of the test article, L-Arabinose Isomerase (EC 5.3.1.4), in terms of clastogenicity using cultured Chinese Hamster Lung (CHL) cells in the presence (+S) and absence (-S) of metabolic activation system. The metabolic activation system was prepared with Aroclor-1254-induced rat liver homogenate (S-9) and cofactor. The test article was prepared with 20 mM Tris-HCl buffer (pH 7.5) provided by the sponsor. The maximum concentration of test article was selected based on the Relative Cell Count (RCC). The dose range was given in the following table. Vehicle control and positive control groups were included, and untreated groups were also set up. Two cultures were used per each dose.

Series	Metabolic activation	Treatment time-Recovery time	Concentration (ug/mL)
1	+	6-18	0
			1,250
			2,500
			5,000
			Untreated
2	-	6-18	0
			1,250
			2,500
			5,000
			Untreated
3	-	24-0	0
			1,250
			2,500
			5,000
			Untreated

Rapidly growing cell cultures were trypsinized and three series of 25 cm² culture flasks (Falcon) were seeded with 6×10^4 cells, each in 5 mL medium, and incubated for about 3 days before the chemical treatment. After 24 hour from the start of treatment, the mitotic cells of each flask were harvested and slides were prepared. A hundred metaphases were evaluated from a slide of each culture, 200 metaphases per concentration in total. The results were presented as counting of structural and numerical aberrations.

There were no statistically significant increases in the frequency of aberrant metaphases in any test article-treated groups compared with the vehicle control group.

In the positive control groups, treated with Benzo[a]pyrene or Ethylmethanesulfonate, clear positive results were obtained.

The test article, L-Arabinose Isomerase (EC 5.3.1.4), did not induce chromosomal aberration in CHL cells which were used in this study. Therefore, L-Arabinose Isomerase (EC 5.3.1.4) is considered to be non-clastogenic.

2. Test, Vehicle and Reference Articles

1) Test article (Annex 2)

- (1) Name: L-Arabinose Isomerase (EC 5.3.1.4)
- (2) Code No. in test facility: C-0441
- (3) Batch No.: CJ-TSAI-0001
- (4) Date of receipt: Feb 17, 2010
- (5) Quantity: (50 mg/mL × 45 mL) / tube × 1 tube
- (6) Appearance: Brown liquid
- (7) Purity: > 95 %
- (8) Concentration of test article: 50 mg/mL
- (9) Expiry date: May 10, 2010
- (10) Storage conditions: Refrigeration
- (11) Supplier: CJ Cheiljedang Food R&D

2) Vehicle

- (1) Name: 20 mM Tris-HCl buffer (pH 7.5)
- (2) Batch No.: CJ-0001-TR
- (3) Supplier: CJ Cheiljedang Food R&D
- (4) Date of receipt: Feb 22, 2010
- (5) Quantity: About 500 mL
- (6) Expiry date: Aug 30, 2010
- (7) Storage conditions: Refrigeration
- (8) Justification for selection: selected by the request of the sponsor.

3) Positive control article

Benzo[a]pyrene (CAS No. 50-32-8, B[a]P) and Ethylmethanesulfonate (CAS No. 62-50-0, EMS) were used as a positive control article in the presence and absence of metabolic activation system, respectively. These positive control articles were listed in the OECD guidelines.

Name	Supplier	Item No	Lot No	Date of receipt	Storage
B[a]P	Sigma-Aldrich Inc (Supelco)	48564	LB60801	Oct 18, 2008	11-30 °C
EMS	Sigma-Aldrich Inc	M0880	1419706	Apr 03, 2009	11-30 °C

3. Materials and Methods

1) Test system

(1) Cells: Chinese hamster lung fibroblasts (CHL/IU cells)

(2) Origin: Chinese Hamster (*Cricetulus griseus*, Female) Lung (Koyama *et al.*, 1970)

(3) Supplier

CHL Cells were obtained from American Type Culture Collection and subcultured in ChemOn Inc. The modal Chromosome number of this cell line is 25 and doubling time is about 15 hours.

(4) Justification for selection

The CHL cell was chosen since it has been widely used for this type of study and there are abundant background data. In addition, this cell line is listed in the aforementioned "OECD Guidelines for the Testing of Chemicals" TG 473.

(5) The cell were stored in liquid nitrogen. The frozen cell was thawed and cultured for more than 7 days, and monitored for contamination with inverted microscope before use for tests. The culture was used for no longer than 3 month after thawing. Examination of mycoplasmal contamination was performed on a regular basis.

(6) Culture medium and cell culture

Cells were cultured in the reconstituted Minimum Essential Medium (MEM, Gibco-BRL #41500-034) supplemented with 2.2 g of sodium bicarbonate, 292 mg of L-glutamine, antibiotics (Penicillin G and Streptomycin sulfate) and 10 % (v/v) fetal bovine serum (FBS, Gibco-BRL) per liter. The medium was filtered with a membrane filter (pore size 0.2 to 0.22 μ m) and supplemented with 10 % (v/v) FBS (Gibco-BRL). During routine culture maintenance, cells were grown as a monolayer in T-75 culture flasks (Falcon) and incubated at 37°C in 5 % CO₂ atmosphere. Cells were subcultured every 2-3 days with 0.1 % trypsin solution.

(7) Cell Preservation

Cells were suspended in the culture medium containing 15 % FBS. One milliliter of DMSO was added to the 9 mL of the cell suspension (Sigma-Aldrich Inc. D-2650), and the mixture was frozen in a cryogenic vial and kept in the liquid nitrogen.

2) Preparation of test article and positive control articles

0 0 0 0 4 8

(1) Test article

The 50 mg/mL test article, supplied by the sponsor, was used as a 10 x stock for the maximum dose. The stocks for the mid- and low-dose levels were prepared, just before use, by diluting the 50 mg/mL liquid with the vehicle supplied by the sponsor. The solution of 50 mg/mL test article and vehicle were filter-sterilized with a syringe filter (25 mm dia.,

CS, cellulose acetate, pore size 0.2 μ m) and kept in an ice bath until use. The solutions were checked for pH using pH strips (Sigma).

(2) Positive control articles

B[a]P was dissolved in DMSO to make 2 mg/mL stock solution and kept at below -50°C before use. EMS was dissolved in the culture medium just before use.

(3) Stability and homogeneity of prepared test article: Not done.

3) Metabolic activation system (S-9 mix)

(1) S-9

Supplier: Molecular Toxicology Inc (P.O. BOX 1189 BOONE, NC 28607, USA).

Origin of S-9: Aroclor 1254-induced rat liver S-9

Species: Sprague-Dawley rat (male)

Item No.: 11-01L

Lot No.: 2493

Protein content: 41.7 mg/mL

Storage condition: in a freezer (below -15°C)

(2) Cofactor

Name: Cofactor-I

Supplier: Wako Pure Chem. Ind., Ltd. (Japan)

Item No.: 309-50611

Lot No.: 999901

Storage condition: Refrigeration (-1 to 10°C)

(3) Composition of S-9 mix (per 1 mL, 30 % S-9 v/v) and use

The composition was made of 8 μ mol $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 33 μ mol KCl, 5 μ mol G-6-P, 4 μ mol NADPH, 4 μ mol NADH, 100 μ mol sodium phosphate buffer (pH 7.4) and 0.3 mL S-9.

(4) S-9 mix was kept on ice for the duration of the experiment.

(5) For metabolic activation, 0.5 mL S-9 mix was added to 2.5 mL treatment mixture/T-25 flask.

4) Dose ranges (Annex 1)

A preliminary range-finding test (Study No: 10-VG-059P) was performed to select dose levels of the present study. In the preliminary study, cells were treated with the same method as present study. After 24 hours from the start of treatment, the cells of each flask were dissociated and counted to calculate Relative Cell Count (RCC) which was regarded as the index of cytotoxicity.

$\text{RCC} = (\text{Cell counts of treated flask} / \text{Cell counts of vehicle control flask}) \times 100 (\%)$

The maximum dose of the present study was selected based on the result of observation at the

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start and end of chemical treatment including formation of precipitation and color change of medium, and RCC at the time of harvest. In the range-finding test cells were treated with a wide range of dose, and there were no precipitation and growth inhibition of cells at 5000 ug/mL, the high dose.

Therefore, in the present study, the the maximum dose was set at 5,000 ug/mL for all series. Treatment series and schedules are as shown in the following table. Vehicle control groups were treated with vehicle, and the untreated groups were treated with culture medium.

[Treatment plan]

	Treatment Groups	S-9 Mix	Dose, ug/mL (volume, ul/flask)	Treatment Schedule (hrs) ^{a)}
1	Vehicle only	+	0 (300)	6 – 18 (designated as "6+S")
	Test article I	+	1,250 (300)	
	Test article II	+	2,500 (300)	
	Test article III	+	5,000 (300)	
	B[a]P	+	20 (30)	
	Untreated	+	Untreated (300)	
2	Vehicle only	-	0 (500)	6 – 18 (designated as "6-S")
	Test article I	-	1,250 (500)	
	Test article II	-	2,500 (500)	
	Test article III	-	5,000 (500)	
	EMS	-	800 (400)	
	Untreated	-	Untreated (300)	
3	Vehicle only	-	0 (500)	24 – 0 (designated as "24-S")
	Test article I	-	1,250 (500)	
	Test article II	-	2,500 (500)	
	Test article III	-	5,000 (500)	
	EMS	-	600 (300)	
	Untreated	-	Untreated (300)	

a) Treatment time-recovery time
 Vehicle: 20 mM Tris-HCl buffer (pH 7.5)
 B[a]P: Benzo [a] pyrene
 EMS: Ethylmethanesulfonate

5) Treatment and preparing specimen

(1) Culture vessel and density

25 cm² culture flasks (Falcon) were seeded with 6 x 10⁴ cells, each in 5 mL medium, and incubated for 3 days.

(2) Identification of cultures

Study number, dose, activation (+S or -S) and treatment time were labeled on the side of flasks.

(3) Duplicate cultures per dose were treated.

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(4) Treatment

Three series of cultures were set up. Before the treatment the old medium of each flask was removed and replaced with 2.2 mL (Series 1) and 4.5 mL (Series 2 and 3) of fresh

medium per flask. The prepared test article was added to each flask at least one hour after the medium change.

The treatment schedules and compositions of treatment mixtures are shown in the chart below.

[Composition of treatment mixture]			
Series	Culture medium + Test article	S-9 mix	Total
1 (6+S)	2.2 mL + 0.3 mL	0.5 mL	3.0 mL
2 (6-S)	4.5 mL + 0.5 mL	-	5.0 mL
3 (24-S)	4.5 mL + 0.5 mL	-	5.0 mL

The treatment mixtures of Series 1 and 2 were aspirated after 6-hour treatment, then cell monolayers were washed with 5 mL CMF D-PBS (Ca^{++} & Mg^{++} free Dulbecco's phosphate buffered saline) and added 5 mL fresh medium, and cultured until collection of mitotic cells. Series 3 was cultured until collection of mitotic cells without washing. Each culture was observed for any abnormal color change or precipitation at the start and end of the treatment.

- (5) Approximately 22 hours after the start of treatment, colchicine solution was added to each culture (final concentration of 1 μM) and incubated for 2 hours.

The mitotic cells were detached by gentle shaking. The medium containing mitotic cells was centrifuged and the cell pellets were resuspended in 75 mM potassium chloride (KCl) solution. Cells were fixed with a fixative (methanol:glacial acetic acid = 3:1 v/v) and slides were prepared by air-drying method. Slides were stained with 5 % Giemsa stain.

- (6) Two slides were prepared for each flask.

- (7) Justification for method of preparation of specimens

This procedure has been widely adopted as a standard method to collect mitotic cells in high density.

- (8) After collecting mitotic cells, the remaining cell monolayers in the flasks were trypsinized and cell counts of each flask was determined using a Coulter Counter for calculation of RCC.

6) Evaluation and presentation of the results

- (1) The morphological identification of chromosome aberration was done according to the principles of 'Atlas of chromosome aberration by chemicals' (JEMS-MMS, 1988).
- (2) A hundred metaphases were evaluated from a selected slide for each culture, 200 metaphases per dose in total.
- (3) Slides were coded before evaluation. Slides were evaluated with a microscope at 1000 X magnification.

(4) Counting of structural aberrations

A hundred metaphases that were well spread and have a chromosome count 23 to 27 were evaluated for aberrations. Aberrations were recorded with its type and the microscope stage co-ordinates.

Aberration was classified into chromosome type deletion/exchange and chromatid type deletion/exchange, and the result was presented both with and without gaps.

(5) Counting of numerical aberrations

After finishing the counting of structural aberrations, regardless of the presence of aberration, an additional 100 metaphases were examined on the same slide to determine the frequencies of diploid (DP, 23-36 chromosomes), polyploid (PP, $37 \leq$ chromosomes) and endoreduplication (ER).

(6) The counted metaphases were classified into normal or aberrant metaphase. The results were presented as numbers of aberrant metaphases with one or more aberrations and total numbers of aberrations per 100 metaphases.

7) Statistics and judgment of the results

According to the OECD guideline, frequencies of aberrant metaphases without gaps were subjected to statistical analyses. The numbers of (PP+ER) were also analyzed. The statistical analyses were performed using SPSS program, and the results was regarded significant if $P < 0.05$.

(1) Comparisons of the vehicle control group with treatment group and of the vehicle control group with positive control group.

Chi-square test and Fisher's exact test

(2) Criteria for evaluation

The result was judged to be positive if there is a statistically significant and dose-related increase in the number of cells with chromosome aberrations. The statistical significance was not regarded as the only determining factor for a positive response, but the biological relevance, frequencies of aberrant metaphases and cytotoxicity were also considered.

(3) Since a positive control article which induces numerical aberration was not used in this study to justify the capability of detection of aneugens, but the number of metaphases with 37 or more chromosomes or endoreduplication was evaluated.

4. Results

1) Results in the presence of S-9 mix (Table 1 and Appendix 1)

There was no precipitation or growth inhibition of cells in all dose levels of test article.

The frequencies of aberrant metaphase were no more than 0.5 per 100 metaphases in the vehicle control and all test article-treated groups, and there were no statistically significant increases in the frequencies of aberrant metaphases in any of the treatment groups when compared to the vehicle control group.

The frequencies of metaphases with numerical aberration were 0.0 per 100 metaphases in the vehicle control and all test article-treated groups.

On the other hand, there was a statistically significant ($P<0.01$) increase in the frequency of aberrant metaphase (31.0) in positive control group when compared to the vehicle control group.

2) Results in the absence of S-9 mix (Table 2 and Appendix 2)

[6 hour-treatment group]

There was no precipitation or growth inhibition of cells in all dose levels of test article.

The frequencies of aberrant metaphase were 0.0 per 100 metaphases in the vehicle control and all test article-treated groups, and there were no statistically significant increases in the frequencies of aberrant metaphases in any of the treatment groups when compared to the vehicle control group.

The frequencies of frequencies of metaphases with numerical aberration were 0.0 per 100 metaphases in the vehicle control and all test article-treated groups.

There was a statistically significant ($P<0.01$) increase in the frequency of aberrant metaphase (13.0) in the positive control group when compared to the vehicle control group.

[24 hour-treatment group]

There was no precipitation or growth inhibition of cells in all dose levels of test article.

The frequencies of aberrant metaphase were no more than 0.5 per 100 metaphases in the vehicle control and all test article-treated groups, and there were no statistically significant increases in the frequency of aberrant metaphases in any of the treatment groups when compared to the vehicle control group.

The frequencies of metaphases with numerical aberration were 0.0 per 100 metaphases in the vehicle control and all test article-treated groups.

There was a statistically significant ($P<0.01$) increase in the frequency of aberrant metaphase (14.5) in positive control group when compared to the vehicle control group.

5. Discussions and Conclusion

This study was carried out to evaluate the mutagenic potential of the test article, L-Arabinose Isomerase (EC 5.3.1.4). For this purpose, the test article was evaluated in terms of clastogenicity using cultured Chinese Hamster Lung (CHL) cells in the presence (+S) and absence (-S) of metabolic activation system.

The metabolic activation system was prepared with Aroclor 1254-induced rat liver homogenate (S-9) and cofactor. The test article was prepared with vehicle.

There was no statistically significant increase in the frequency of aberrant metaphases at any dose level of test article, when compared with the vehicle control group, regardless of the presence or absence of metabolic activation system or treatment time.

In the positive control groups, treated with Benzo[a]pyrene or Ethylmethanesulfonate, clear positive results were obtained.

Between the vehicle control and untreated groups there was no difference in the frequency of aberrant metaphase or cell proliferation, and this demonstrates that the vehicle did not have harmful effect on the performance of the study.

The test article, L-Arabinose Isomerase (EC 5.3.1.4), did not induce chromosomal aberration in CHL cells which were used in this study. Therefore, L-Arabinose Isomerase (EC 5.3.1.4) is considered to be non-clastogenic.

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6. References

1. Korea Food and Drug Administration: Good Laboratory Practice, Notification No. 2009-183 (Dec 22, 2009).
2. Korea Food and Drug Administration: the Standards of Toxicity Study for Medicinal Products, Notification No. 2009-116 (Aug 24, 2009).
3. NIER: Good Laboratory Practice Standards and Test Guidelines, Notification No. 2009-57 (Nov 24, 2009).
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8. OECD (1997): OECD Guideline for Testing of Chemicals (July 21, 1997) TG473 '*In Vitro* Mammalian Chromosomal Aberration Test'.
9. OECD (1997): OECD Principles on Good Laboratory Practice.

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7. Tables (group summaries)

Table 1. Chromosome aberration test in the presence of S-9 mix^{a)}

Dose of Test Article (ug/mL) ^{b)}	Treatment Schedule (hours) ^{c)}	Mean Aberrant Metaphases ^{d)}		Mean total Aberrations ^{d)}		Mean of PP + ER	RCC (%)
0		1.0 /	0.5	1.0 /	0.5	0.0 + 0.0	100
1,250		0.0 /	0.0	0.0 /	0.0	0.0 + 0.0	104
2,500	6 - 18	0.0 /	0.0	0.0 /	0.0	0.0 + 0.0	98
5,000		0.5 /	0.0	0.5 /	0.0	0.0 + 0.0	103
B[a]P 20		32.5 /	31.0 ^{**e)}	51.0 /	44.0	0.0 + 0.5	69
untreated		0.0 /	0.0	0.0 /	0.0	0.0 + 0.0	101

****** Significantly different from the control at $P < 0.01$

Test article: L-Arabinose Isomerase (EC 5.3.1.4)

a) See Appendix 1 and 3 for individual data.

b) Nominal concentration of the Test Article.

c) Treatment time - recovery time.

d) Gaps included/excluded, means of duplicate cultures.
100 metaphases were examined per culture.

e) Fisher's exact test.

PP: Polyploid

ER: Endoreduplication

B[a]P: Benzo[a]Pyrene

RCC: Relative Cell Counts = (Cell counts of treated flask/ Cell counts of vehicle control flask) x 100 (%)

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Table 2. Chromosome aberration test in the absence of S-9 mix^{a)}

Dose of Test Article (ug/mL) ^{b)}	Treatment Schedule (hours) ^{c)}	Mean Aberrant Metaphases ^{d)}		Mean total Aberrations ^{d)}		Mean of PP + ER	RCC (%)
0	6 - 18	0.5 /	0.0	0.5 /	0.0	0.0 + 0.0	100
1,250		0.0 /	0.0	0.0 /	0.0	0.0 + 0.0	102
2,500		0.0 /	0.0	0.0 /	0.0	0.0 + 0.0	101
5,000		0.5 /	0.0	0.5 /	0.0	0.0 + 0.0	100
EMS 800		15.0 /	13.0 ** ^{e)}	19.5 /	15.0	0.0 + 0.0	73
untreated		0.5 /	0.5	0.5 /	0.5	0.0 + 0.0	102
0	24 - 0	0.0 /	0.0	0.0 /	0.0	0.0 + 0.0	100
1,250		0.0 /	0.0	0.0 /	0.0	0.0 + 0.0	98
2,500		1.0 /	0.5	1.0 /	0.5	0.0 + 0.0	102
5,000		0.5 /	0.0	0.5 /	0.0	0.0 + 0.0	96
EMS 600		17.5 /	14.5 ** ^{e)}	21.5 /	17.0	0.0 + 0.0	71
untreated		1.0 /	0.0	1.0 /	0.0	0.0 + 0.0	104

** Significantly different from the control at $P < 0.01$

Test article: L-Arabinose Isomerase (EC 5.3.1.4)

a) See Appendix 2 and 3 for individual data.

b) Nominal concentration of the Test Article.

c) Treatment time - recovery time.

d) Gaps included/excluded, means of duplicate cultures.

100 metaphases were examined per culture.

e) Fisher's exact test.

PP: Polyploid

ER: Endoreduplication

EMS: Ethylmethanesulfonate

RCC: Relative Cell Counts = (Cell counts of treated flask/ Cell counts of vehicle control flask) x 100 (%)

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8. Appendices (individual data)

Appendix 1. Chromosome aberration test in the presence of S-9 mix

Dose of Test Article (ug/mL)	Treatment Schedule (hours) ^{a)}	Number of Aberrant Metaphases ^{b)}	Number of Total Aberrations ^{b)}	Number of findings/100 metaphases						
				Gap	Chromosome Type		Chromatid Type		Other	PP + ER
					DEL	EXC	DEL	EXC		
0 (A)	6 - 18	1 / 0	1 / 0	1	0	0	0	0	0	0 + 0
0 (B)		1 / 1	1 / 1	1	0	0	0	2	0	0 + 0
1,250 (A)		0 / 0	0 / 0	0	0	0	0	0	0	0 + 0
1,250 (B)		0 / 0	0 / 0	0	0	0	0	0	0	0 + 0
2,500 (A)		0 / 0	0 / 0	0	0	0	0	0	0	0 + 0
2,500 (B)		0 / 0	0 / 0	0	0	0	0	0	0	0 + 0
5,000 (A)		0 / 0	0 / 0	0	0	0	0	0	0	0 + 0
5,000 (B)		0 / 0	0 / 0	0	0	0	0	0	0	0 + 0
B[a]P 20 (A)		28 / 27	51 / 41	10	0	0	3	32	6	0 + 1
B[a]P 20 (B)		37 / 35	51 / 47	4	0	0	2	37	8	0 + 0
untreated (A)		0 / 0	0 / 0	0	0	0	0	0	0 + 0	
untreated (B)		0 / 0	0 / 0	0	0	0	0	0	0 + 0	

Test article: L-Arabinose Isomerase (EC 5.3.1.4)

a) Treatment time-recovery time.

b) Gaps included/excluded, 100 metaphases per culture.

DEL: Deletion

EXC: Exchange

PP: Polyploid

ER: Endoreduplication

B[a]P: Benzo [a] Pyrene

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Appendix 2. Chromosome aberration test in the absence of S-9 mix

Dose of Test Article (ug/mL)	Treatment Schedule (hours) ^{a)}	Number of Aberrant Metaphases ^{b)}	Number of Total Aberrations ^{b)}	Number of findings/100 metaphases						
				Chromosome Type		Chromatid Type		Other	PP + ER	
				Gap	DEL	EXC	DEL	EXC		
0 (A)	6 - 18	0 / 0	0 / 0	0	0	0	0	0	0	0 + 0
0 (B)		1 / 0	1 / 0	1	0	0	0	0	0	0 + 0
1,250 (A)		0 / 0	0 / 0	0	0	0	0	0	0	0 + 0
1,250 (B)		0 / 0	0 / 0	0	0	0	0	0	0	0 + 0
2,500 (A)		0 / 0	0 / 0	0	0	0	0	0	0	0 + 0
2,500 (B)		0 / 0	0 / 0	0	0	0	0	0	0	0 + 0
5,000 (A)		0 / 0	0 / 0	0	0	0	0	0	0	0 + 0
5,000 (B)		1 / 0	1 / 0	1	0	0	0	0	0	0 + 0
EMS 800 (A)		13 / 12	19 / 15	4	0	0	0	15	0	0 + 0
EMS 800 (B)		17 / 14	20 / 15	5	0	0	0	15	0	0 + 0
untreated (A)	24 - 0	1 / 1	1 / 1	0	0	0	0	1	0	0 + 0
untreated (B)		0 / 0	0 / 0	0	0	0	0	0	0	0 + 0
0 (A)		0 / 0	0 / 0	0	0	0	0	0	0	0 + 0
0 (B)		0 / 0	0 / 0	0	0	0	0	0	0	0 + 0
1,250 (A)		0 / 0	0 / 0	0	0	0	0	0	0	0 + 0
1,250 (B)		0 / 0	0 / 0	0	0	0	0	0	0	0 + 0
2,500 (A)		0 / 0	0 / 0	0	0	0	0	0	0	0 + 0
2,500 (B)		2 / 1	2 / 1	1	0	0	0	1	0	0 + 0
5,000 (A)		1 / 0	1 / 0	1	0	0	0	0	0	0 + 0
5,000 (B)		0 / 0	0 / 0	0	0	0	0	0	0	0 + 0
EMS 600 (A)		18 / 14	22 / 16	6	0	0	2	14	0	0 + 0
EMS 600 (B)		17 / 15	21 / 18	3	0	0	4	14	0	0 + 0
untreated (A)		1 / 0	1 / 0	1	0	0	0	0	0	0 + 0
untreated (B)		1 / 0	1 / 0	1	0	0	0	0	0	0 + 0

Test article: L-Arabinose Isomerase (EC 5.3.1.4)

a) Treatment time-recovery time.

b) Gaps included/excluded, 100 metaphases per culture.

DEL: Deletion

EXC: Exchange

PP: Polyploid

ER: Endoreduplication

EMS: Ethylmethanesulfonate

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Appendix 3. Cell counts of main study^{a)}

Dose of Test Article (ug/mL)	S-9 mix	Treatment Schedule (hours) ^{b)}	Cell Counts				Mean	Relative Cell Counts (%) ^{c)}
			Flask A		Flask B			
0	+	6 - 18	7500	7399	7573	7394	7467	100
1,250	+		7802	7881	7797	7710	7798	104
2,500	+		7233	7113	7469	7537	7338	98
5,000	+		7757	7808	7685	7571	7705	103
B[a]P 20	+		5185	5142	5043	5164	5134	69
untreated	+		7694	7599	7445	7542	7570	101
0	-	6 - 18	8439	8169	8196	8247	8263	100
1,250	-		8506	8549	8411	8401	8467	102
2,500	-		8413	8482	8207	8263	8341	101
5,000	-		8360	8238	8197	8119	8229	100
EMS 800	-		5965	5903	6146	6090	6026	73
untreated	-		8638	8693	8233	8101	8416	102
0	-	24 - 0	7328	7233	7742	7521	7456	100
1,250	-		7256	7138	7688	7284	7342	98
2,500	-		7677	7699	7532	7515	7606	102
5,000	-		7059	6996	7229	7328	7153	96
EMS 600	-		5436	5379	5153	5236	5301	71
untreated	-		7855	7719	7697	7618	7722	104

Test article: L-Arabinose Isomerase (EC 5.3.1.4)

a) Two flasks/dose were used. After harvesting mitotic cells, each culture was trypsinized and suspended with 0.5 mL of 0.1% trypsin and 5 mL of culture medium. The cell suspensions of 0.4 mL/culture was diluted 50 times with 19.6 mL of Isoton® sol. (Beckman Coulter Co.). The cells in 0.5 mL Isoton® sol. were counted twice/culture using Coulter Counter model Z2. Actual number of cells per flask = Mean Count × 550.

b) Treatment time - recovery time.

c) RCC: Relative Cell Counts = (Cell counts of treated flask/ Cell counts of vehicle control flask) x 100 (%)

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9. Annexes

Annex 1. Result of preliminary range-finding test

Annex 2. Certificate of analysis (test article)

Annex 3. Historical control data

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Annex 1. Result of preliminary range-finding test^{a)}

Dose of Test Article (ug/mL)	S-9 mix	Treatment Schedule (hours) ^{b)}	Cell Counts		Mean	Relative Cell Counts (%) ^{c)}
0	+	6 - 18	8473	8186	8330	100
10	+		8149	8206	8178	98
100	+		8206	8383	8295	100
1,000	+		8219	8041	8130	98
1,500	+		7809	7728	7769	93
2,500	+		8035	7912	7974	96
5,000	+		7837	7936	7887	95
0	-	6 - 18	8679	8884	8782	100
10	-		8873	8643	8758	100
100	-		8136	8054	8095	92
1,000	-		8045	8060	8053	92
1,500	-		8448	8380	8414	96
2,500	-		8655	8791	8723	99
5,000	-		8143	8075	8109	92
0	-	24 - 0	8465	8480	8473	100
10	-		8642	8445	8544	101
100	-		8640	8310	8475	100
1,000	-		8357	8172	8265	98
1,500	-		8083	8046	8065	95
2,500	-		8237	8084	8161	96
5,000	-		8527	8517	8522	101

Test article: L-Arabinose Isomerase (EC 5.3.1.4)

a) One flask/dose were used. After harvesting mitotic cells, each culture was trypsinized and suspended with 0.5 mL of 0.1% trypsin and 5 mL of culture medium. The cell suspensions of 0.4 mL/culture was diluted 50 times with 19.6 mL of Isoton[®] sol. The cells in 0.5 mL Isoton[®] sol. were counted twice/culture using Coulter Counter model Z2. Actual number of cells per flask = Mean Count × 550.

b) Treatment time - recovery time.

c) RCC: Relative Cell Counts = (Cell counts of treated flask/ Cell counts of vehicle control flask) x 100 (%)

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Annex 2. Certificate of analysis (test article)

Statement of Study Director

Title: Product sheet of L-Arabinose Isomerase (EC 5.3.1.4)

(b) (6)



March 10, 2010

Young-Ho Hong Ph.D.

Date

Study director

Test Facility : CJ Cheiljedang Food R&D

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Annex 2. Certificate of analysis (test article)

Product Sheet

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L-Arabinose Isomerase (EC 5.3.1.4)

Batch NO. CJ-TSAI-0001

Description

L-Arabinose Isomerase is a heat-stable isomerization enzyme from *Thermotoga neapolitana* expressed in and produced by a *Corynebacterium glutamicum*.

Product properties

Enzyme class	L-Arabinose Isomerase (EC 5.3.1.4)
Molecular weight (KDa)	226 (consisted 4 subunit in 56 KDa)
Declared activity	9.5 U/mg (based optimal condition)
Color	Brown
Physical form	Liquid
Stability	See the Fig. 1.
Solubility	50 mg/ml in water or buffer
Appro Density (mg/ml)	50
Stabilizer	Mn ²⁺ , Co ²⁺ , Mg ²⁺
Production organism	<i>Thermotoga neapolitana</i> Produced by liquid fermentation of <i>Corynebacterium glutamicum</i> . The enzyme protein is separated and purified the fermented organism.
Product purity (%)	> 95

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Annex 2. Certificate of analysis (test article)

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Product characters

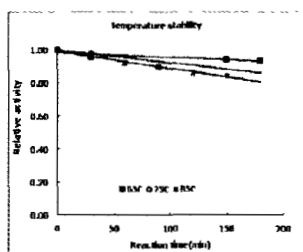


Fig. 1. Thermostability on L-Arabinose Isomerase.

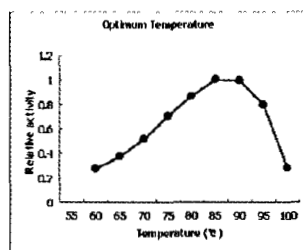


Fig. 2. The effect of temperature on L-Arabinose Isomerase activity.

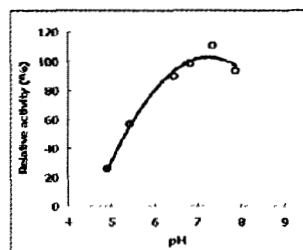


Fig. 3. The effect of pH on L-Arabinose Isomerase activity

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Annex 2. Certificate of analysis (test article)

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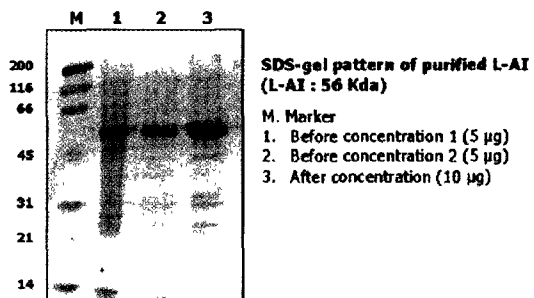


Fig. 4. The SDS-PAGE pattern of purified L-Arabinose Isomerase.

Storage

Recommended storage conditions are 0-10°C in unbroken packaging. The product has been formulated for optimal stability with 20 mM Tris-HCl (pH 7.5). However, enzymes gradually lose activity over time.

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Annex 3. Historical control data

Chinese Hamster Lung cells, all vehicles, 2005–2010.

Negative controls

Treatment method ^{a)}	S-9 mix	% Aberrant metaphase of structural aberration (Range)	N
6 – 18	+	0.12 ± 0.34 (0 - 2)	130
	-	0.14 ± 0.37 (0 - 2)	130
24 – 0	-	0.05 ± 0.21 (0 - 1)	130

a) Treatment time-recovery time

Positive controls

Treatment method ^{a)} (article & conc.) ^{b)}	S-9 mix	% Aberrant metaphase of structural aberration (Range)	N
6 – 18 (B[a]P, 20 ug/mL)	+	23.83 ± 6.12 (14 - 35)	12
6 – 18 (EMS, 800 ug/mL)	-	22.10 ± 8.16 (9 - 51)	128
24 – 0 (EMS, 600 ug/mL)	-	24.19 ± 10.69 (9 – 58)	128

a) Treatment time-recovery time

b) B[a]P: Benzo[a]pyrene [50-32-8], EMS: Ethylmethanesulfonate [62-50-0]

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FINAL REPORT

**A Single-Dose oral toxicity of L-Arabinose
Isomerase (EC 5.3.1.4) in ICR Mice with Fixed
Dose Procedure**

Study Number: 10-MA-056

Sponsor: CJ Cheiljedang Food R&D

Preclinical Research Center, ChemOn Inc.



Preclinical Research Center, Chemon Inc.
334, Jeil-ri, Yangji-myeon, Cheoin-gu, Yongin-si, Gyeonggi-do,
449-826, Korea

000068

Translation Verification Statement

This report is a translation of original Korean Final Report issued on May 19, 2010.
Hereby, I certify that the contents are accurately translated.

(b) (6)



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Study director
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July 22, 2010

Date

000069

Statement of Management

Title: A Single-Dose oral toxicity of L-Arabinose Isomerase (EC 5.3.1.4) in ICR Mice with Fixed Dose Procedure

The above-mentioned study is carried out according to the guidelines of GLP and laboratory studies.

1. National Institute of Environmental Research: Good Laboratory Practice, Notification No. 2009-57 (Nov 24, 2009).
2. Korea Food and Drug Administration: The Standards of Toxicity Study for Medicinal Products, Notification No. 2009-116 (Aug 24, 2009).
3. Korea Food and Drug Administration: Good Laboratory Practice Regulation for Nonclinical Laboratory Studies, Notification No. 2009-183 (Dec 22, 2009).
4. OECD Principles of Good Laboratory Practice (1997)

(Signature in the original report)

Chan-Koo Park, DVM, M.S.

Management

Preclinical Research Center of ChemOn Inc

May 19, 2010

Date

000070

Statement of Study Director

Title: A Single-Dose oral toxicity of L-Arabinose Isomerase (EC 5.3.1.4) in ICR Mice with Fixed Dose Procedure

The above-mentioned study is carried out according to the guidelines of GLP and laboratory studies.

1. National Institute of Environmental Research: Good Laboratory Practice, Notification No. 2009-57 (Nov 24, 2009)
2. Korea Food and Drug Administration: The Standards of Toxicity Study for Medicinal Products, Notification No. 2009-116 (Aug 24, 2009).
3. Korea Food and Drug Administration: Good Laboratory Practice Regulation for Nonclinical Laboratory Studies, Notification No. 2009-183 (Dec 22, 2009).
4. OECD Principles of Good Laboratory Practice (1997))

(Signature in the original report)

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May 19, 2010

Date

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Quality Assurance Statement

Study number: 10-MA-056

Title: A Single-Dose oral toxicity of L-Arabinose Isomerase (EC 5.3.1.4) in ICR Mice with Fixed Dose Procedure

Study period: Feb 25, 2010 – May 19, 2010

Sponsor: CJ Cheiljedang Food R&D

Items	Inspected on	Inspection results confirmed to Study Director on	Inspection results reported to Management on
Protocol	Feb 24, 2010	Feb 25, 2010	Feb 26, 2010
Animal receipt/Quarantine	Mar 04, 2010	Mar 04, 2010	Mar 05, 2010
Storage of test/reference article	Mar 11, 2010	Mar 11, 2010	Mar 12, 2010
	Mar 18, 2010	Mar 18, 2010	Mar 19, 2010
Preparation of test/reference article	Mar 11, 2010	Mar 11, 2010	Mar 12, 2010
	Mar 18, 2010	Mar 18, 2010	Mar 19, 2010
Administration and animal care	Mar 11, 2010	Mar 11, 2010	Mar 12, 2010
	Mar 18, 2010	Mar 18, 2010	Mar 19, 2010
Observation and examination	Mar 15, 2010	Mar 15, 2010	Mar 15, 2010
	Mar 25, 2010	Mar 26, 2010	Mar 26, 2010
Necropsy	Apr 01, 2010	Apr 01, 2010	Apr 02, 2010
Raw data	Apr 21, 2010	Apr 29, 2010	Apr 29, 2010
Final report (draft)	Apr 21, 2010	Apr 29, 2010	Apr 29, 2010
Final report	May 19, 2010	-	-

Hereby, I do certify that the detailed methods, procedures, and observations in this report are accurately and completely described, and that the reported results accurately and completely reflect the raw data, and that this study was performed in conformity with National Institute of Environmental Research: The Standards of Toxicity Study for Chemical Products, Annex. 5 of Notification No. 2009-57 (Nov 24, 2009), the Korea Food and Drug Administration(KFDA) Notification No. 2009-116 'The Standards of Toxicity Study for Medicinal Products' (Aug 24, 2009), The KFDA Notification No. 2009-183 'Good Laboratory Practice Regulation for Nonclinical Laboratory Studies'(Dec 22, 2009), National Institute of Environmental Research: Good Laboratory Practice, Notification No. 2009-57 (Nov 24, 2009). and OECD Principles of Good Laboratory Practice(1997).

Date: May 19, 2010

(Signature in the original report)

Kap-Ho Kim, M.S.
Quality Assurance Manager,
Preclinical Research Center, ChemOn Inc.

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Synopsis

Title	A Single-Dose oral toxicity of L-Arabinose Isomerase (EC 5.3.1.4) in ICR Mice with Fixed Dose Procedure
Objectives	The present study was carried out to evaluate the toxicity of L-Arabinose Isomerase (EC 5.3.1.4) in ICR mice after a single intravenous administration with Fixed Dose Procedure.
Sponsor	Name: CJ Cheiljedang Food R&D Address:: 636 Guro-dong, Guro-gu, Seoul(150-050), Korea Managing Director: Young-Ho Hong Contact: +82-2-2629-5336 (TEL), +82-2-2629-5334 (FAX)
Test Facility	Name: Preclinical Research Center, ChemOn Inc. Address: 334 Jeil-ri, Yangji-myeon, Cheoin-gu, Yongin-si, Gyeonggi-do, 449-826, Korea Management: Chan-Koo Park Contact: +82-31-329-9900 (TEL), +82-31-329-9901 (FAX)
Schedule	Feb 25, 2010: Approval of protocol (study initiation) Mar 04, 2010: Animal Acquisition (Experimental initiation) Mar 11, 2010: Initiation of pre-administration Mar 18, 2010: Initiation of administration Apr 01, 2010: Necropsy Apr 26, 2010: Submission of draft report May 19, 2010: Submission of final report (Study completion)
Archives	The protocol and amendments, final report, raw data, specimens, sample of test article, and relevant documents produced during the present study period will be retained and stored in the Archives of Preclinical Research Center, ChemOn Inc. for three years after study completion. Further storage of above materials shall be consulted with the Sponsor.

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Contributing Scientists

DIVISIONS	NAME	DUTIES
Animal Tests	Joo-Young Lee Laboratory Animal Technician	Chief of Animal experiment
Storage / Preparation of the test article	Hyun-Suk Heo	Chief of Preparation and Storage of test article
Necropsy	Hak-Soo Kim Laboratory Animal Technician, Medical and clinical laboratory technologist	Chief of Necropsy
Statistical analysis	Min-Haeng Lee, M.S.	Chief of Statistical analysis
Archives	Jin- Young Lee	Chief of Archives

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1. Summary

The present study was carried out to evaluate the toxicity of L-Arabinose Isomerase (EC 5.3.1.4) in ICR mice after a single oral administration with Fixed Dose Procedure. As a sighting study, one male and female per group were administered the test article at doses of 300 and 2,000 mg/kg. After observations including mortality and clinical signs, the doses of this study were set. In this study, test article was administered orally once at doses of 0 (vehicle control) and 2,000 mg/kg to 10 animals per group (5 males and 5 females). Observations included mortality, clinical signs, body weight and necropsy findings, and the results were compared with those of the vehicle control group. Followings are the summary of the results.

1. There was no death during the experimental period.
2. In clinical signs, there were no signs attributable to the test article.
3. Body weights of animals in all treatment groups increased similarly to those of the vehicle control group during the experimental period.
4. In the necropsy findings, there were no test article-related findings.

Based on the aforementioned results, following the single-dose oral administration of L-Arabinose Isomerase (EC 5.3.1.4) in ICR mice with L-Arabinose Isomerase (EC 5.3.1.4) Fixed Dose Procedure (FDP), no dead animal was observed. Therefore, Approximate Lethal Dose of L-Arabinose Isomerase (EC 5.3.1.4) in mice was considered to be higher than 2,000 mg/kg and is ranked as a Category 5 (GHS).

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2. Test article and vehicle

1) Test article

- (1) Name: L-Arabinose Isomerase (EC 5.3.1.4)
- (2) Code No. in test facility: C-0441
- (3) Batch Number: CJ-TSAI-0001
- (4) Date of receipt: Feb 17, 2010
- (5) Amount: 50 mg/mL X 45 mL/tube X 1 tube
- (6) Appearance: Brown liquid
- (7) Purity: > 95 %
- (8) Content of test article: 50 mg/mL
- (9) Expiration date: May 10, 2010
- (10) Storage: Refrigeration
- (11) Supplier: CJ Cheiljedang Food R&D

2) Solution

- (1) Name: 20 mM Tris-HCl buffer (pH 7.5)
- (2) Batch Number: CJ-0001-TR
- (3) Supplier : CJ Cheiljedang Food R&D
- (4) Date of receipt: Feb 22, 2010
- (5) Amount: approximately 500 mL
- (6) Expiration date: Aug 30, 2010
- (7) Storage conditions: Refrigeration
- (8) Justification of selection: The vehicle is selected by request of the sponsor.

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3. Materials and Methods

1) Test system

- (1) Species and strain: Specific pathogen free (SPF) mice, HsdKoat:ICR(CD-1[®])
- (2) Provider: Koatech Co. Ltd (406 Dongcheon-ri, Jinwi-myeon, Pyeongtaek-si, Gyeonggi-do, Korea, TEL: +82-31-611-8222)
- (3) Justification of selection

The ICR mice used in this study are suitable because they are generally used for the toxicity test and the basic data for toxicity test have been accumulated in abundance, therefore the data can be used to interpret and evaluate the test results. Fixed Dose Procedure (FDP) is generally conducted using a single sex (female). But both males and females were selected, for test article to be exposed to males and females.

- (4) The range of ages and weights

Age, sex, and number at receipt:

7 weeks of age, male and female rats, 13 males and 13 females

Weight ranges at receipt: male 30.63-33.21 g, female 24.56-26.75 g

Age, sex and number of animals at administration: 8-9 weeks of age, male and female rats, 12 males and 12 females

Weight ranges at administration

[1st sighting study] male 33.78 g, female 26.49 g

[2nd sighting study] male 34.19 g, female 27.83 g

[Main study] male 34.20-37.21 g, female 27.25-32.17 g

- (5) Quarantine and acclimation (Annex 2)

On arrival, animal's external appearances were examined, according to the certificate provided by the supplier and were acclimated for 7 days (1st sighting study), 8 days (2nd sighting study) and 14 days (Main study) in the animal room where the present study was performed. During the acclimation period, only healthy animals were selected for the further experiment.

2) Husbandry

- (1) Environmental conditions

This study was performed within Room No. 3 in the animal facility area No. 2 of Preclinical Research Center, ChemOn Inc., and the animals were housed in a room that was maintained at a temperature of 23±3 °C and a relative humidity of 55±15 %, with artificial lighting from 08:00 to 20:00, 150-300 Lux of luminous intensity and 10-20 air changes per hour. All the researchers wore autoclaved (121 °C, 20 min.) working clothes and special protective equipments during the testing.

Throughout the study period, the temperature and humidity of animal room were measured

every hour with a computer-based automatic sensor, and the environmental conditions such as ventilation frequency and luminous intensity were monitored on a regular basis. As a result of environmental measurements, the room was maintained at a temperature of 22.2-22.8 °C, and a relative humidity of 58.7-61.2 %, and there were no deviations that could affect this study.

(2) Cages, housing density and cage identification

During the quarantine and acclimatization, 5 animals or less were housed in a stainless steel wire mesh cage (165 W x 240 L x 145 H mm), and during administration and observation periods, 3 animals or less were housed in the same type of cage. In males, one animal was housed in the same type of cage during the study.

(3) Diet and water (Annex 3 and 4)

Animals were offered irradiation-sterilized pellet food for lab animal (Harlan Co. Ltd, USA. TEKLAD CERTIFIED GLOBAL 18% PROTEIN RODENT DIET, 2918C) purchased from Koatech Co. Ltd. Underground water disinfected by ultraviolet sterilizer and ultrafiltration was given via water bottle, *ad libitum*.

According to the certificates on diet components and contaminants supplied by diet provider, there were no factors that could affect results of this study.

Examination of water was performed by an authorized Gyeonggido Institute of Health & Environment (324-1, Pajang-dong, Jangan-gu, Suwon-si, Gyeonggido, Korea), and there were no factors that could affect results.

(4) Animal experimentation ethics

The present study was performed on the basis of the Animal Experimentation Policy of ChemOn Inc according to the 'animal protection laws' legislate; No. 4379(May 31, 1991), reform; No. 8852(Feb 29, 2008) (serial number: 10-M013).

3) Dosage and grouping

(1) Group identification

Group	Sex	Number of animal	Identification of animal	Volume(ml/kg)	Dose(mg/kg)
1 st sighting study	M / F	1 / 1	1 / 2	40	300
2 nd sighting study	M / F	1 / 1	3 / 4	40	2,000
Main study ^{a)}	M / F	5 / 5	1-5 / 11-15	40	0
Main study	M / F	5 / 5	6-10 / 16-20	40	2,000

a) Vehicle control [20 mM Tris-HCl buffer (pH 7.5)]

(2) Selection of dose levels

There was no toxic information on L-Arabinose Isomerase (EC 5.3.1.4). Therefore, the dose of 1st sighting study was selected with the starting dose level of 300 mg/kg which was recommended by the 'OECD Guideline No. 425' and 'National Institute of Environmental

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Research'. No dead animals or toxicological signs were observed. The dose of 2nd sighting study was selected at 2,000 mg/kg, and no dead animals and toxicological signs were observed. Therefore, the dose of main study was selected at 2,000 mg/kg and the control group was received vehicle only.

(3) Grouping and disposition of the remnant animals

Grouping was done as follows. Healthy animals selected during the acclimatization period were weighed and animals whose body weight was close to the average weight were selected. And then, referring to the rank of body weight, these animals were distributed randomly so that animals of each group were distributed uniformly according to the "group identification" table. The remnant animals were administered euthanasia.

(4) Identification

Animals were identified by a tail marking method using the oil pen during the quarantine/acclimation periods, and individually distinguished by fur marking using saturated picric acid solution in absolute ethanol and ear punch during the administration and observation periods. Cages are individually identified by color-coded ID cards. Cage racks were given serial numbers and a log sheet of use was attached at the entrance of the animal room including the study number, study title, housing period, name and emergency contact number of study director and name of study personnel.

4) Preparation of test article

The test article was weighed without compensation before preparation. The formulation of 2,000 mg/kg dose was used in unprocessed form without further preparation. In the formulation of 300 mg/kg dose, the test article was weighed and the final volume was adjusted with the vehicle. The test article was freshly prepared just before administration.

5) Administration

(1) Justification for the route of administration:

The oral administration was selected as the intended clinical route for the test article.

(2) Route and method of administration

The test article for dosing was administered directly into the stomach using a feeding needle with syringe tube. Before the administration, animals were fasted during 3~4 hours. Food was offered about 2 hours after administration.

(3) Volume of administration

Animals were dosed at dose volumes of 40 (less than 2000 mg/kg) and 50 ml/kg (2000 mg/kg), and individual volume for each rat is calculated based on the body weight measured on the day of administration.

(4) Frequency and period of administration

Twice a day, animals of the 300 and 2000 mg/kg dose groups were dosed twice at dose

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volumes of 20 and 25 mL/kg at 2~3 hour interval, respectively. The administration was completed by 15:00. The day of the first dosing will be designated as day 0.

6) Items of observation and examination

(1) Clinical signs and mortality

[Sighting study]

The clinical signs and mortalities of administered animals on the administration day were observed continuously for the first 30 minutes after administration and then, every hour for 4 consecutive hours afterward. The observation of clinical signs was performed once a day after administration until Day 14. The results are presented in the appendix section.

[Main study]

The clinical signs and mortalities of all animals on the administration day were observed continuously for the first 30 minutes after administration and then, every hour for 4 consecutive hours afterward. The observation of clinical signs was performed once a day after administration until Day 14.

(2) Body weights

[Sighting study]

Animals were individually weighed before administration (Day 0), and on days 1, 3, 7 and 14 after administration..

[Main study]

Animals were individually weighed before administration (Day 0), and on days 1, 3, 7 and 14 after administration..

(3) Necropsy

On day 14 after administration, all animals were anesthetized by inhaling CO₂, and then after laparotomy, were terminated by exsanguination from the posterior vena cava and abdominal aorta. All the findings in the examination of body surface and organs in the abdomen and thoracic cavities will be recorded.

7) Statistical analysis

The data of body weights were expressed as mean \pm SD and were subjected to student's t-test to compare the treatment groups. Significance will be judged at a probability value of $p \leq 0.05$. The commercial statistics program, SPSS 10.1K software, was used for statistical analyses.

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4. Results

1) Mortality (Table 1 and Appendix 1-1~1-4)

No dead animal was observed.

Female no. 14 in the vehicle control group was found escaped from the cage at Day 1, therefore the animal was sacrificed at the discretion of the study director.

2) Clinical signs (Table 2 and Appendix 1-1~1-4)

There were no clinical signs during the experimental period

3) Body weights (Table 3 and Appendix 2-1~ 2-3)

Body weights of animals in all treatment groups increased similar to those of the vehicle control group during the experimental period.

4) Necropsy findings (Table 4 and Appendix 3)

No notable findings were recorded in the treatment groups

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5. Discussion and Conclusion

The present study was carried out to evaluate the toxicity of L-Arabinose Isomerase (EC 5.3.1.4) in ICR mice after a single intravenous administration with Fixed Dose Procedure.

In results, no dead animal was observed and no test article-related changes in body weights and necropsy findings were observed.

Based on the aforementioned results, following the single-dose oral administration of L-Arabinose Isomerase (EC 5.3.1.4) in ICR mice with L-Arabinose Isomerase (EC 5.3.1.4) Fixed Dose Procedure(FDP), no dead animal was observed. Therefore, Approximate Lethal Dose of L-Arabinose Isomerase (EC 5.3.1.4) in mice was considered to be higher than 2,000 mg/kg and is ranked as a Category 5 (GHS).

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6. Tables (group summaries)

Table 1. Mortalities of mice

STUDY: 10-MA-056		DAYS AFTER DOSE									SEX: MALE	
GROUPS (mg/kg)	No. DEAD/ No. DOSED	0	1	2	3	4	5	6	7	8-14	ALD ^{a)} Value	GHS category
G1 (0)	0 / 5	0	0	0	0	0	0	0	0	0		
G2 (2,000)	0 / 5	0	0	0	0	0	0	0	0	0	2,000 mg/kg	5
SEX: FEMALE												
G1 (0)	0 / 4 ^{b)}	0	0	0	0	0	0	0	0	0		
G2 (2,000)	0 / 5	0	0	0	0	0	0	0	0	0	2,000 mg/kg	5

a) ALD: Approximate Lethal Dose.

b) 1 animal escaped the cage.

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Table 2. Clinical signs of mice

STUDY: 10-MA-056		CLINICAL SIGNS			
DAYS	SIGNS	GROUPS (mg/kg)			
		MALE		FEMALE	
		G1 (0)	G2 (2,000)	G1 (0)	G2 (2,000)
0	Normal	5 / 5 ^{a)}	5 / 5	5 / 5	5 / 5
1	Normal	5 / 5	5 / 5	4 / 5	5 / 5
	Euthanasia ^{b)}	0 / 5	0 / 5	1 / 5	0 / 5
2-13	Normal	5 / 5	5 / 5	4 / 4	5 / 5
14	Normal	5 / 5	5 / 5	4 / 4	5 / 5
	Terminal sacrifice	5 / 5	5 / 5	4 / 4	5 / 5

a) Number of animals with the sign/Number of animals examined.

b) 1 animal escaped the cage.

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Table 3. Body weight changes of mice

BODY WEIGHTS (g)				
STUDY: 10-MA-056		GROUPS (mg/kg)		
	MALE		FEMALE	
DAYS	G1 (0)	G2 (2,000)	G1 (0)	G2 (2,000)
DAY 0	35.15±0.74 (5) ^{b)}	35.79±1.15 (5)	29.99±1.90 (5)	29.41±1.69 (5)
DAY 1	35.48±1.62 (5)	35.15±1.28 (5)	29.45±1.97 (4) ^{c)}	30.02±2.24 (5)
DAY 4	36.35±1.43 (5)	36.19±1.81 (5)	29.66±2.21 (4) ^{c)}	30.48±2.24 (5)
DAY 7	36.42±1.64 (5)	36.37±1.86 (5)	30.05±1.11 (4) ^{c)}	30.65±2.25 (5)
DAY 14	37.00±1.33 (5)	37.79±1.97 (5)	31.07±1.80 (4) ^{c)}	32.31±2.97 (5)
GAINS ^{a)}	1.85±0.80 (5)	2.00±0.95 (5)	1.43±1.34 (4) ^{c)}	2.90±1.43 (5)

a) Weight gains are body weight difference between day 14 and the day 0.

b) Number of animals.

c) 1 animal escaped the cage.

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Table 4. Necropsy findings of mice

STUDY: 10-MA-056		NECROPSY FINDINGS		SEX: MALE	
GROUPS (mg/kg)	LOCATION	FINDINGS	FREQUENCY ^{a)}		
			DEAD	ALIVE	
G1 (0)		No gross findings	0 / 0	5 / 5	
G2 (2,000)		No gross findings	0 / 0	5 / 5	
SEX: FEMALE					
G1 (0)		No gross findings	0 / 0	4 / 4 ^{b)}	
G2 (2,000)		No gross findings	0 / 0	5 / 5	

a) Number of animals with the sign/Number of animals examined.

b) 1 animal escaped the cage.

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7. Appendices (individual data)

Appendix 1-1. Clinical signs of mice (1st sighting study)

STUDY: 10-MA-056		CLINICAL SIGNS	
(mg/kg)	ANIMAL ID	SIGNS	OBSERVED ON ^{a)}
(300)	1	Normal	DAY 0-14
		Terminal sacrifice	DAY 14
	2	Normal	DAY 0-14
		Terminal sacrifice	DAY 14

Appendix 1-2. Clinical signs of mice (2nd sighting study)

STUDY: 10-MA-056		CLINICAL SIGNS	
(mg/kg)	ANIMAL ID	SIGNS	OBSERVED ON ^{a)}
(2,000)	3	Normal	DAY 0-14
		Terminal sacrifice	DAY 14
	4	Normal	DAY 0-14
		Terminal sacrifice	DAY 14

Appendix 1-3. Clinical signs of male mice (main study)

STUDY: 10-MA-056		CLINICAL SIGNS		SEX: MALE
GROUPS (mg/kg)	ANIMAL ID	SIGNS	OBSERVED ON ^{a)}	
G1 (0)	1	Normal	DAY 0-14	
		Terminal sacrifice	DAY 14	
	2	Normal	DAY 0-14	
		Terminal sacrifice	DAY 14	
	3	Normal	DAY 0-14	
		Terminal sacrifice	DAY 14	
	4	Normal	DAY 0-14	
		Terminal sacrifice	DAY 14	
	5	Normal	DAY 0-14	
		Terminal sacrifice	DAY 14	
G2 (2,000)	6	Normal	DAY 0-14	
		Terminal sacrifice	DAY 14	
	7	Normal	DAY 0-14	
		Terminal sacrifice	DAY 14	
	8	Normal	DAY 0-14	
		Terminal sacrifice	DAY 14	
	9	Normal	DAY 0-14	
		Terminal sacrifice	DAY 14	
	10	Normal	DAY 0-14	
		Terminal sacrifice	DAY 14	

a) The day of administration was designated as day 0.

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Appendix 1-4. Clinical signs of female mice (main study)

STUDY: 10-MA-056			SEX: FEMALE
GROUPS (mg/kg)	ANIMAL ID	SIGNS	OBSERVED ON ^{a)}
G1 (0)	11	Normal	DAY 0-14
		Terminal sacrifice	DAY 14
	12	Normal	DAY 0-14
		Terminal sacrifice	DAY 14
	13	Normal	DAY 0-14
		Terminal sacrifice	DAY 14
	14	Normal	DAY 0
		Euthanasia ^{b)}	DAY 1
G2 (2,000)	15	Normal	DAY 0-14
		Terminal sacrifice	DAY 14
	16	Normal	DAY 0-14
		Terminal sacrifice	DAY 14
	17	Normal	DAY 0-14
		Terminal sacrifice	DAY 14
	18	Normal	DAY 0-14
		Terminal sacrifice	DAY 14
	19	Normal	DAY 0-14
		Terminal sacrifice	DAY 14
	20	Normal	DAY 0-14
		Terminal sacrifice	DAY 14

a) The day of administration was designated as day 0.

b) Animal escaped the cage.

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Appendix 2-1. Body weight changes of mice (1st sighting study)

STUDY: 10-MA-056		BODY WEIGHTS (g)			
(mg/kg)	ANIMAL ID	Day 0	Day 1	Day 4	GAINS ^{a)}
(300)	1	33.78	34.22	34.16	0.38
	2	26.49	27.17	28.67	2.18

a) Weight gains are body weight difference between day 14 and the day 0.

Appendix 2-2. Body weight changes of mice (2nd sighting study)

STUDY: 10-MA-056		BODY WEIGHTS (g)			
(mg/kg)	ANIMAL ID	Day 0	Day 1	Day 4	GAINS ^{a)}
(2,000)	3	34.19	34.99	34.39	0.20
	4	27.83	28.80	27.42	-0.41

a) Weight gains are body weight difference between day 14 and the day 0.

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Appendix 2-3. Body weight changes of mice (main study)

STUDY: 10-MA-056		BODY WEIGHTS (g)					SEX: MALE
GROUPS (mg/kg)	ANIMAL ID	Day 0	Day 1	Day 4	Day 7	Day 14	GAINS ^{a)}
G1 (0)	1	34.20	33.25	34.81	34.55	36.04	1.84
	2	35.04	34.57	35.49	35.26	35.79	0.75
	3	34.91	35.58	35.71	36.07	36.36	1.45
	4	36.25	36.79	38.07	38.29	38.84	2.59
	5	35.33	37.21	37.65	37.92	37.97	2.64
	MEAN	35.15	35.48	36.35	36.42	37.00	1.85
	S.D.	0.74	1.62	1.43	1.64	1.33	0.80
	N	5	5	5	5	5	5
G2 (2,000)	6	34.79	34.29	35.29	35.14	36.46	1.67
	7	34.47	34.33	34.27	33.99	35.06	0.59
	8	36.00	35.29	36.41	37.55	39.10	3.10
	9	36.49	34.54	35.88	36.51	38.53	2.04
	10	37.21	37.32	39.10	38.67	39.79	2.58
	MEAN	35.79	35.15	36.19	36.37	37.79	2.00
	S.D.	1.15	1.28	1.81	1.86	1.97	0.95
	N	5	5	5	5	5	5
STUDY: 10-MA-056		BODY WEIGHTS (g)					SEX: FEMALE
GROUPS (mg/kg)	ANIMAL ID	Day 0	Day 1	Day 4	Day 7	Day 14	GAINS ^{a)}
G1 (0)	11	27.25	28.06	28.45	29.91	30.50	3.25
	12	29.33	28.62	28.12	28.74	29.77	0.44
	13	29.87	28.73	29.17	30.09	30.29	0.42
	14	31.38	-	-	-	-	-
	15	32.11	32.37	32.91	31.44	33.73	1.62
	MEAN	29.99	29.45	29.66	30.05	31.07	1.43
	S.D.	1.90	1.97	2.21	1.11	1.80	1.34
	N	5	4	4	4	4	4
G2 (2,000)	16	27.57	28.59	29.06	28.28	29.28	1.71
	17	29.10	28.78	29.32	29.20	31.76	2.66
	18	28.90	29.89	30.31	30.21	32.48	3.58
	19	29.31	28.90	29.30	31.49	30.85	1.54
	20	32.17	33.92	34.39	34.07	37.17	5.00
	MEAN	29.41	30.02	30.48	30.65	32.31	2.90
	S.D.	1.69	2.24	2.24	2.25	2.97	1.43
	N	5	5	5	5	5	5

a) Weight gains are body weight difference between day 14 and the day 0.

-: Animal escaped the cage, and then euthanasia.

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Appendix 3. Necropsy findings of mice

STUDY: 10-MA-056				SEX: MALE
GROUPS (mg/kg)	ANIMAL ID	FATE	LOCATION	FINDINGS
G1 (0)	1	Terminal sacrifice		No gross findings
	2	Terminal sacrifice		No gross findings
	3	Terminal sacrifice		No gross findings
	4	Terminal sacrifice		No gross findings
	5	Terminal sacrifice		No gross findings
G2 (2,000)	6	Terminal sacrifice		No gross findings
	7	Terminal sacrifice		No gross findings
	8	Terminal sacrifice		No gross findings
	9	Terminal sacrifice		No gross findings
	10	Terminal sacrifice		No gross findings
				SEX: FEMALE
GROUPS (mg/kg)	ANIMAL ID	FATE	LOCATION	FINDINGS
G1 (0)	11	Terminal sacrifice		No gross findings
	12	Terminal sacrifice		No gross findings
	13	Terminal sacrifice		No gross findings
	14	Euthanasia		-
	15	Terminal sacrifice		No gross findings
G2 (2,000)	16	Terminal sacrifice		No gross findings
	17	Terminal sacrifice		No gross findings
	18	Terminal sacrifice		No gross findings
	19	Terminal sacrifice		No gross findings
	20	Terminal sacrifice		No gross findings

-: Animal escaped the cage, and then euthanasia.

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8. Annexes

Annex 1-1. Certificate of test item

Statement of Study Director

Title: Product sheet of L-Arabinose Isomerase (EC 5.3.1.4)

(b) (6)



March 10, 2010

Young-Ho Hong Ph.D.

Date

Study director

Test Facility : CJ Cheiljedang Food R&D

Address : 636, Guro-dong, Guro-gu, Seoul, 150-050, Korea

Contact : +82-2-2629-5336 (TEL.), +82-2-2629-5344 (FAX)

E-mail : gene9695@cj.net

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Annex 1-2. Certificate of test item

Product Sheet

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L-Arabinose Isomerase (EC 5.3.1.4)

Batch NO. CJ-TSA1-0001

Description

L-Arabinose Isomerase is a heat-stable isomerization enzyme from *Thermotoga neapolitana* expressed in and produced by a *Corynebacterium glutamicum*

Product properties

Enzyme class	L-Arabinose Isomerase (EC 5.3.1.4)
Molecular weight (KDa)	226 (consisted 4 subunit in 56 KDa)
Declared activity	9.5 U/mg (based optimal condition)
Color	Brown
Physical form	Liquid
Stability	See the Fig 1.
Solubility	50 mg/ml in water or buffer
Appro. Density (mg/ml)	50
Stabilizer	Mn ²⁺ , Co ²⁺ , Mg ²⁺
Production organism	<i>Thermotoga neapolitana</i> Produced by liquid fermentation of <i>Corynebacterium glutamicum</i> . The enzyme protein is separated and purified the fermented organism.
Product purity (%)	> 95

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Annex 1-3. Certificate of test item

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Product characters

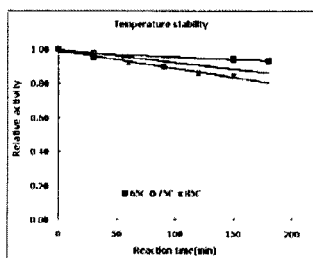


Fig. 1 Thermostability on L-Arabinose Isomerase.

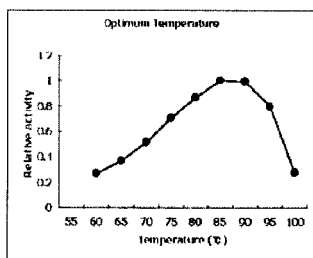


Fig. 2. The effect of temperature on L-Arabinose Isomerase activity.

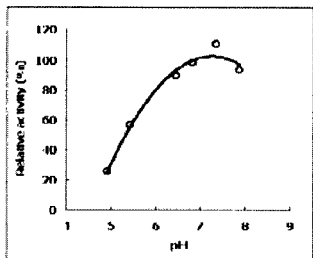


Fig. 3. The effect of pH on L-Arabinose Isomerase activity

000095

Annex 1-4. Certificate of test item

Page 3/3

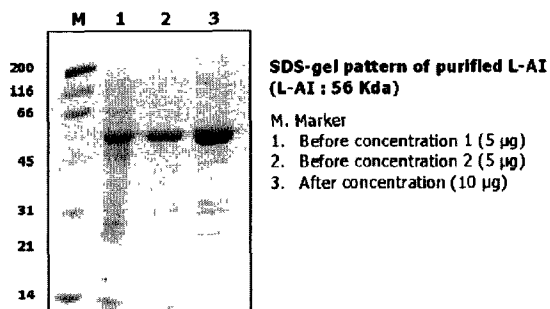


Fig. 4. The SDS-PAGE pattern of purified L-Arabinose Isomerase.

Storage

Recommended storage conditions are 0~10℃ in unbroken packaging. The product has been formulated for optimal stability with 20 mM Tris-HCl (pH 7.5). However, enzymes gradually lose activity over time.

000096

Results of Microbiological Monitoring

M10030-2

Contributor : 코아텍

Room No.:A103

Species: Mouse

Strain: Hsd:ICR(CD-1®)

● Results

검사 방법	Date of request(여섯주)	2008. 7. 28		2008. 10. 25		2009. 1. 29			2009. 4. 29		2009. 7. 28			2009. 10. 28		2010. 1. 26	
		3.4	8.10	3.4	8.10	3.4	8.10	11	3.4	8.10	3.4	8.10	11	3.4	8.10	3.4	8.10
		weeks	weeks	weeks	weeks	weeks	weeks	month	weeks	weeks	weeks	weeks	month	weeks	weeks	weeks	weeks
Microbes		(4)	(4)	(4)	(4)	(2)	(2)	(2)	(2)	(2)	(2)	(2)	(2)	(2)	(2)	(2)	(4)
		NT*	0/4	NT*	0/4	0/2	0/3	0/2	0/1	0/2	0/1	0/2	0/3	0/1	0/4	0/1	0/1
항 진 사	Mouse parvovirus	NT*	0/4	NT*	0/4	0/2	0/3	0/2	0/1	0/2	0/1	0/2	0/3	0/1	0/4	0/1	0/1
	Reovirus 3	NT*	0/4	NT*	0/4	0/1	0/3	0/2	0/1	0/2	0/1	0/2	0/3	0/1	0/4	0/1	0/1
	Rotavirus(Epzootic diarrhea of infant mice)	NT*	0/1	NT*	0/1	0/1	0/3	0/2	0/1	0/2	0/1	0/2	0/3	0/1	0/4	0/1	0/1
	Sendai virus (HVJ)	NT*	0/4	NT*	0/4	0/1	0/3	0/2	0/1	0/2	0/1	0/2	0/3	0/1	0/4	0/1	0/1
	Mouse hepatitis virus (MHV)	NT*	0/4	NT*	0/4	0/1	0/3	0/2	0/1	0/2	0/1	0/2	0/3	0/1	0/4	0/1	0/1
	Ectromelia virus (Mouse pox)	NT*	0/4	NT*	0/4	0/1	0/3	0/2	0/1	0/2	0/1	0/2	0/3	0/1	0/4	0/1	0/1
	Mycoplasma spp	NT*	0/4	NT*	0/4	0/1	0/3	0/2	0/1	0/2	0/1	0/2	0/3	0/1	0/4	0/1	0/1
	Clostridium piliforme (Tyzzer's disease)	NT*	0/4	NT*	0/4	0/1	0/3	0/2	0/1	0/2	0/1	0/2	0/3	0/1	0/4	0/1	0/1
	Mouse adenovirus (MAV)	NT*	0/4	NT*	0/4	0/1	0/3	0/2	0/1	0/2	0/1	0/2	0/3	0/1	0/4	0/1	0/1
	Hanta virus	NT*	0/4	NT*	0/4	0/1	0/3	0/2	0/1	0/2	0/1	0/2	0/3	0/1	0/4	0/1	0/1
	Pneumonia virus of mice (PVM)	NT*	0/4	NT*	0/4	0/1	0/3	0/2	0/1	0/2	0/1	0/2	0/3	0/1	0/4	0/1	0/1
	Mouse encephalomyelitis virus (GD VII)	NT*	0/4	NT*	0/4	0/1	0/3	0/2	0/1	0/2	0/1	0/2	0/3	0/1	0/4	0/1	0/1
	Minute virus of mice (MVM)	NT*	0/4	NT*	0/4	0/1	0/3	0/2	0/1	0/2	0/1	0/2	0/3	0/1	0/4	0/1	0/1
	Lymphocytic choriomeningitis (LCM) virus	NT*	0/4	NT*	0/4	0/1	0/3	0/2	0/1	0/2	0/1	0/2	0/3	0/1	0/4	0/1	0/1
	Cilia associated respiratory(CAR) bacillus	NT*	0/4	NT*	0/4	0/1	0/3	0/2	0/1	0/2	0/1	0/2	0/3	0/1	0/4	0/1	0/1
항 진 사	Pasteurella pneumotropica	0/4	0/4	0/4	0/4	0/2	0/3	0/2	0/2	0/2	0/2	0/2	0/3	0/2	0/4	0/2	0/4
	Salmonella spp	0/4	0/4	0/4	0/4	0/2	0/3	0/2	0/2	0/2	0/2	0/2	0/3	0/2	0/4	0/2	0/4
	Ecoli O157a(K/B)	0/4	0/4	0/4	0/4	0/2	0/3	0/2	0/2	0/2	0/2	0/2	0/3	0/2	0/4	0/2	0/4
	Corynebacterium kutscheri	0/4	0/4	0/4	0/4	0/2	0/3	0/2	0/2	0/2	0/2	0/2	0/3	0/2	0/4	0/2	0/4
	Pseudomonas aeruginosa	0/4	0/4	0/4	0/4	0/2	0/3	0/2	0/2	0/2	0/2	0/2	0/3	0/2	0/4	0/2	0/4
	Staphylococcus aureus	0/4	0/4	0/4	0/4	0/2	0/3	0/2	0/2	0/2	0/2	0/2	0/3	0/2	0/4	0/2	0/4
	Dermatophytes	0/4	0/4	0/4	0/4	0/2	0/3	0/2	0/2	0/2	0/2	0/2	0/3	0/2	0/4	0/2	0/4
	Giardia muris	0/4	0/4	0/4	0/4	0/2	0/3	0/2	0/2	0/2	0/2	0/2	0/3	0/2	0/4	0/2	0/4
검 정	Spironucleus muris	0/4	0/4	0/4	0/4	0/2	0/3	0/2	0/2	0/2	0/2	0/2	0/3	0/2	0/4	0/2	0/4
	Trichomonas spp	0/4	0/4	0/4	0/4	0/2	0/3	0/2	0/2	0/2	0/2	0/2	0/3	0/2	0/4	0/2	0/4
	Syphacia spp	0/4	0/4	0/4	0/4	0/2	0/3	0/2	0/2	0/2	0/2	0/2	0/3	0/2	0/4	0/2	0/4
	Entamoeba spp	0/4	0/4	0/4	0/4	0/2	0/3	0/2	0/2	0/2	0/2	0/2	0/3	0/2	0/4	0/2	0/4
	Ectoparasite	0/4	0/4	0/4	0/4	0/2	0/3	0/2	0/2	0/2	0/2	0/2	0/3	0/2	0/4	0/2	0/4
	Helicobacter hepaticus	0/4	0/4	0/4	0/4	0/2	0/3	0/2	0/2	0/2	0/2	0/2	0/3	0/2	0/4	0/2	0/4
PCR	Helicobacter bilis	0/4	0/4	0/4	0/4	0/2	0/3	0/2	0/2	0/2	0/2	0/2	0/3	0/2	0/4	0/2	0/4

● Remark : 검사를 의뢰한 마우스의 헬스 모니터링 결과 상기 항목에 대해 모든 동물이 음성결과 나타

(b) (6)

Lab. of Animal Model Evaluation

Bio-Evaluation Center

Korea Research Institute of Bioscience and Biotechnology

Hyoung-Chin Kim, DVM, Ph. D.

ICLAS Monitoring Subcenter Korea

685-1 Yangcheon-gu Ochang-myeon Cheongwon-gun Chungbuk Korea

☎ 82-43-240-6560 Fax 82-43-240-6569 E-mail: hckim@kribb.re.kr

* 본 결과는 코아텍이 한국생명공학연구원 에 의뢰하여 검정한 결과입니다 NT: not tested

000097

ChemCon Inc.

22

10-MA-056

Annex 3-1. Laboratory diet certification report

Laboratory Diet Certification Report**2918C**Lot Number **2918C-070709MA**Date of Manufacture **07/07/09**Report Date **07/21/09**

The following data is a consolidation of results obtained from one or more independent testing laboratories. The actual laboratory results are available upon request.

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2009.07.21
14:01:56 -05'00'

[Signature]

Director

Analysis	Result (%)
Protein	18.60
Fat	6.11
Fiber	3.80
Moisture	11.60
Ash	5.28
Calcium	1.00
Phosphorus	0.71

Analysis	Result	Units	Established Maximum Concentration
Heavy Metals			
Arsenic	0.18	ppm	1.00
Cadmium	< 0.10	ppm	0.50
Lead	< 0.20	ppm	1.50
Mercury	< 0.05	ppm	0.20
Selenium	0.25	ppm	0.50
Mycotoxin			
Aflatoxin B1, B2, G1, G2	< 5.00	ppb	5.00
Chlorinated Hydrocarbons			
Aldrin	< 0.01	ppm	0.03
Lindane	< 0.01	ppm	0.05
Chlordane	< 0.01	ppm	0.05
DDT & related substances	< 0.03	ppm	0.15
Dieldrin	< 0.02	ppm	0.03
Endrin	< 0.02	ppm	0.03
Heptachlor	< 0.01	ppm	0.03
Heptachlor Epoxide	< 0.01	ppm	0.03
Toxaphene	< 0.10	ppm	0.15
PCB's	< 0.10	ppm	0.15
a-BHC	< 0.01	ppm	0.05
b-BHC	< 0.01	ppm	0.05
d-BHC	< 0.01	ppm	0.05
Hexachlorobenzene	< 0.01	ppm	0.03
Mirex	< 0.01	ppm	0.02
Methoxychlor	< 0.05	ppm	0.50
Organophosphates			
Thimet	< 0.15	ppm	0.50
Diazinon	< 0.14	ppm	0.50
Disulfoton	< 0.15	ppm	0.50
Methyl Parathion	< 0.14	ppm	0.50
Malathion	< 0.14	ppm	0.50
Parathion	< 0.12	ppm	0.50
Thiodan	< 0.02	ppm	0.50
Ethion	< 0.14	ppm	0.50
Trithion	< 0.15	ppm	0.50

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SPECIALTY LABORATORIES, INC.

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Annex 3-2. Laboratory diet certification report

Laboratory Diet Certification Report**2918C**Lot Number **2918C-080409MA**Date of Manufacture **08/04/09**Report Date **08/14/09**

The following data is a consolidation of results obtained from one or more independent testing laboratories. The actual laboratory results are available upon request.

(b) (6)

I have reviewed this document

2009.08.17 10:12:27

-05'00'

Quality Assurance Coordinator Technical Data
Research, Modeling and Services
Harlan Laboratories, Inc.

Analysis	Result (%)
Protein	17.90
Fat	5.96
Fiber	3.89
Moisture	12.00
Ash	5.34
Calcium	0.90
Phosphorus	0.70

Analysis	Result	Units	Established Maximum Concentration
Heavy Metals			
Arsenic	0.13	ppm	1.00
Cadmium	< 0.10	ppm	0.50
Lead	< 0.20	ppm	1.50
Mercury	< 0.05	ppm	0.20
Selenium	0.24	ppm	0.50
Mycotoxin			
Aflatoxin B1, B2, G1, G2	< 5.00	ppb	5.00
Chlorinated Hydrocarbons			
Aldrin	< 0.01	ppm	0.03
Lindane	< 0.01	ppm	0.05
Chlordane	< 0.01	ppm	0.05
DDT & related substances	< 0.03	ppm	0.15
Dieldrin	< 0.02	ppm	0.03
Endrin	< 0.02	ppm	0.03
Heptachlor	< 0.01	ppm	0.03
Heptachlor Epoxide	< 0.01	ppm	0.03
Toxaphene	< 0.10	ppm	0.15
PCB's	< 0.10	ppm	0.15
a-BHC	< 0.01	ppm	0.05
b-BHC	< 0.01	ppm	0.05
d-BHC	< 0.01	ppm	0.05
Hexachlorobenzene	< 0.01	ppm	0.03
Mirex	< 0.01	ppm	0.02
Methoxychlor	< 0.05	ppm	0.50
Organophosphates			
Thimet	< 0.15	ppm	0.50
Diazinon	< 0.14	ppm	0.50
Disulfoton	< 0.15	ppm	0.50
Methyl Parathion	< 0.14	ppm	0.50
Malathion	< 0.14	ppm	0.50
Parathion	< 0.12	ppm	0.50
Thiodan	< 0.02	ppm	0.50
Ethion	< 0.14	ppm	0.50
Tnthon	< 0.15	ppm	0.50

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Annex 4. Report of water analysis



경기도보건환경연구원

수신자:

경기 용인시 처인구 양지면 제일리 334 (주)캠온 연구지원팀

(경유)

제목:

수질검사성적서

접 수 번 호	1150902365-02	시 료 명	지하수46항	의뢰 일자	2009년 12월 7일
의뢰 지	최태규	의뢰 주소	경기 용인시 처인구 양지면 제일리334 (주)캠온 연구지원팀		
채 취 장 소	경기 용인시 처인구 양지면 제일리 334 (주)캠온 - 제2동분사유구역 1~9호실	임 회 공 무 원			
성 적 서 용 도	참고	시 험 한 검	온도 12.1℃, pH 7.1, DO 1.0mg/L, 탁도 0.1NTU	시 험 기 간	2009-12-07~2009-12-16
시 험 방 법	먹는물수질공정시험기준				
1. 시험의 결과는 검사목적 이외의 광고, 선전, 용기포장 등에 이를 사용 또는 표시할 수 없음.					
2. 참고용은 관계공무원이 봉합봉인하지 않은 시료로서 수질검사성적서는 제출 및 기타증빙서류로 사용할 수 없음					

검사항목	기준	결과	단위	검사항목	기준	결과	단위
일반세균	100이하	0	CFU/mL	1,1-디클로로에틸렌	0.03이하	불검출	mg/L
총대장균군	불검출	불검출	/100mL	디클로로메탄	0.02이하	불검출	mg/L
분원성대장균군	불검출	불검출	/100mL	1,1,1-트리클로로에탄	0.1이하	불검출	mg/L
납	0.05이하	불검출	mg/L	사염화탄소	0.002이하	불검출	mg/L
불소	1.5이하	불검출	mg/L	트리클로로에틸렌	0.03이하	불검출	mg/L
비소	0.05이하	불검출	mg/L	테트라클로로에틸렌	0.01이하	불검출	mg/L
세레늄	0.01이하	불검출	mg/L	1,2-디브로모-3-클로로프로판	0.003이하	불검출	mg/L
수은	0.001이하	불검출	mg/L	경도	300이하	85.1	mg/L
시안	0.01이하	불검출	mg/L	과망간산칼륨 소비량	10이하	불검출	mg/L
6가크롬	0.05이하	불검출	mg/L	냄새	이취 없을것	적합	-
암모니아성질소	0.5이하	불검출	mg/L	맛	이미 있을것	적합	-
질산성질소	10이하	2.2	mg/L	구리	1.0이하	불검출	mg/L
카드뮴	0.005이하	불검출	mg/L	색도	5이하	1	도
보론	1.0이하	불검출	mg/L	세제	0.5이하	불검출	mg/L
베를	0.005이하	불검출	mg/L	수소이온농도	5.8~8.5	6.6	-
다이아지논	0.02이하	불검출	mg/L	아린	3.0이하	0.0167	mg/L
파라티온	0.06이하	불검출	mg/L	염소이온	250이하	14	mg/L
페니트로치온	0.04이하	불검출	mg/L	중발산류물	500이하	112.0	mg/L
카바릴	0.07이하	불검출	mg/L	질	0.30이하	불검출	mg/L
벤젠	0.01이하	불검출	mg/L	망간	0.3이하	불검출	mg/L
톨루엔	0.7이하	불검출	mg/L	탁도	1이하	0.056	NTU
에틸벤젠	0.3이하	불검출	mg/L	황산이온	200이하	8	mg/L
크실렌	0.5이하	불검출	mg/L	암모니아	0.2이하	불검출	mg/L
판정	적합 : 「판정은 의뢰된 항목에 한함」						

경기도보건환경연구원



지방환경연구사 김문정

지방환경연구관 최양희

구부장

최승석

함조자

시행

수질연구부-12586

2009년 12월 16일

우 440-290 경기도 수원시 장안구 파장동 이몽골길 13(파장동 324-1) / <http://www.kihe.re.kr>

전화 031-250-2574

전송 031-250-2654

사용자 ibuddha@gg.go.kr

000100

Pages 000101 - 000118 have been removed in accordance with copyright laws. Please see appended bibliography list of the references that have been removed from this request.

Mr Andreas Klepsch
European Commission
By email

15 August 2005

Reference: NFU 537

Dear Mr Klepsch,

INITIAL OPINION: D-TAGATOSE

On 1 March 2005, the UK Competent Authority accepted an application from Bioresco Ltd, on behalf of Arla Food Ingredients (Denmark) for D-Tagatose as a novel food ingredient, in accordance with Article 4 of regulation (EC) 258/97. The Advisory Committee on Novel Foods and Processes (ACNFP) reviewed this application and their opinion is attached. I apologise for the delay in submitting this opinion as the ACNFP's evaluation was extended while we obtained additional information from the applicant.

In view of the ACNFP's opinion, the UK Competent Authority considers that D-Tagatose meets the criteria for acceptance of a novel food defined in Article 3(1) of Regulation (EC) 258/97.

I am copying this letter and the ACNFP's opinion to the applicant.

Yours sincerely,

Dr Chris Jones
For the UK Competent Authority

000119

ADVISORY COMMITTEE ON NOVEL FOODS AND PROCESSES

**OPINION ON AN APPLICATION UNDER THE NOVEL
FOODS REGULATION FOR D-TAGATOSE**

UK OPINION

000120

ADVISORY COMMITTEE FOR NOVEL FOODS AND PROCESSES

OPINION ON AN APPLICATION UNDER THE NOVEL FOODS REGULATION FOR D-TAGATOSE

Applicant	Bioresco, on behalf of Arla Foods, Denmark
Responsible Person	Dr Albert Bär
EC Classification	2.1

Introduction

1. An application has been submitted by Bioresco, acting on behalf of Arla Food Ingredients, Denmark for authorisation of D-tagatose as a novel ingredient in the EU.
2. D-tagatose is a monosaccharide, an enantiomer of D-fructose (inversion at C-4), which is not commonly found in food, although it is found at low levels in heat-treated dairy products such as sterilised and dried milk. D-tagatose has 75-92% the sweetness of sucrose and behaves like other sugars in terms of hygroscopicity, and stability under low pH and raised temperature. Its principal purpose is as a carbohydrate source, with purported nutritional effects of non-cariogenicity and as a prebiotic. During preliminary discussions with the applicant, the Secretariat noted that the use of D-tagatose in foods could fall within the legal definition of a sweetener, requiring authorisation under food additive legislation rather than the regulation on novel foods. This issue has been resolved following discussion with the Commission and other MS and the consensus view is that tagatose should be regarded as a novel food ingredient and not as a food additive.
3. This opinion details the safety of this novel ingredient and does not investigate or comment on the perceived nutritional effects that the applicant attributes to its consumption.

I. Specification of the novel food

Information on this aspect is provided on pp 14-16 and pp25-27, Annexes 1, 3 and 4 of the application dossier

4. As an enantiomer of D-fructose, D-tagatose has the empirical formula $C_6H_{12}O_6$ (see Figure 1). An overview of the compositional analyses of D-tagatose and the raw materials used in its production are given in Annex 1, sections 3 and 5. Detailed information on the specifications of raw materials, process chemicals and ion exchange resins are listed in Annex 1.

5. The novel ingredient (NI) is synthesised by enzymatic hydrolysis from lactose with a purity of $\geq 99\%$. All chemicals used in the production process are high purity and have low levels of heavy metals (Annex 1). The resulting D-tagatose has a purity of no less than 98%, a lead content no greater than 1 ppm and an ash content of no more than 0.1%.
6. D-tagatose is produced from lactose using a two-step process. In the first instance lactose is enzymically hydrolysed to galactose and glucose. The galactose is then isomerised to D-tagatose at a high pH using calcium hydroxide as a complexing agent.
7. Batch-on-batch variation has been determined by analysis of 6 batches of D-tagatose, produced by the applicant at pilot scale (Annex 4). These indicate a high degree of reproducibility. HPLC data (Annex 4) show that the only detectable impurity in the final product is galactose, which is present as a by-product of the production process.
8. D-tagatose has been evaluated by JECFA¹ on three occasions, most recently in 2004 when it allocated an ADI "not specified"². The detail of the toxicological evaluation by JECFA is discussed later in this paper. The JECFA specification for D-tagatose is given in Annex 3.

Discussion Members were satisfied with the specification of the novel food.

II. Effect of the production process applied to the novel food

Information on this aspect is provided on pp 17 – 24 of the application dossier

9. D-tagatose is produced from food-grade lactose by a two-stage process involving enzymatic hydrolysis of food-grade lactose to form galactose, which then isomerises to D-tagatose under alkaline conditions. The applicant has summarised the process on p17 and included a detailed flow diagram (Figure 2).
10. All chemicals used in the production process including the raw material (lactose) and the immobilised lactase (obtained from *Aspergillus oryzae*) are food grade, as are all anti-microbials and column regeneration chemicals.

11. Process

Lactose is first dissolved in hot water and the pH is adjusted, by addition of lactose solution that has been passed through an ion exchange column, to obtain a mildly acidic solution. This solution is then pasteurised before being passed through a column that contains immobilised lactase. This enzyme preparation is widely used throughout the EU. To avoid contamination, the column is regularly treated with a defined anti-microbial solution.

¹ JECFA: Joint FAO/WHO Expert Group on Food Additives.

² ADI Not Specified: Used by JECFA to refer to a food substance of low toxicity which on the basis of the available data, the total dietary exposure necessary to achieve the desired effect, and acceptable background levels in food does not represent a hazard to health.

12. The resultant hydrolysed lactose solution is concentrated by evaporation before being fractionated using a cation exchange resin. The resultant fractions are collected and the galactose-rich fraction retained. This fraction is cooled and the galactose is converted to D-tagatose by addition of a defined amount of Ca(OH)_2 , which moves the isomerisation equilibrium in favour of the D-tagatose. D-tagatose is precipitated as an insoluble complex with calcium. Once this stage is completed the NI is removed and re-dissolved by addition of CO_2 which neutralises the mixture and causes precipitation of the calcium as CaCO_3 .

13. Purification

The NI is purified by filtration, evaporation, demineralisation, and fractionation. These are described in detail on pages 20-22 of the application dossier.

14. The applicant notes that the conditions used to produce the NI are relatively benign and do not favour other reactions that could potentially occur, particularly during the isomerisation of D-galactose. A brief discussion of the potential impurities that could arise as a result of the occurrence of these 'side reactions' is detailed on page 25. None of the compounds described were found in detectable quantities in the end product (Annex 4).

Discussion Members were content that the production process employed by the applicant does not give rise to concern

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

Information on this aspect is provided on pp 33-46 and Annex 6 of the application dossier

15. The applicant intends the NI to be used as a nutritive ingredient in a variety of products. The availability of these products will not be restricted geographically and there are no plans to target these products at particular consumer groups. A list of products and the levels at which D-tagatose is typically expected to be added can be found in the table below:

Food Category	Proposed food use	Added Tagatose (g per 100g of food)
Baked goods	Cookies	2
	Quick breads	2
	Muffins	2
	Quick bread type	2
	Coffee cakes	2
Beverages	Diet" and "sugar- free" carbonated beverages; non- carbonated Beverages sweetened with low- calorie sweeteners – includes milk-based beverages, juices, juice drinks, teas, and coffee- based Beverages (ready- to- drink, prepared from mix, and dry mix forms)	1
Coffee drinks	Such as cappuccino and latte	1
Frozen milk based desserts, reduced/low fat	Light ice cream	3
	Frozen milk desserts	3
	Low fat and non fat frozen yoghurts	3

	Related frozen novelties	3
Hard candies	Hard candies including regular and dietetic candies	15
Health bars and diet soft candies	Low fat, reduced fat, diet meal, energy or nutrient fortified bars, dietetic soft candies	10
Icings	Icings (or glazes), such as those used on cookies, pastries, brownies, and angel food, chiffon, and pound cakes	30
Meal Replacement / supplement Beverages	Meal replacement beverages, diet meal beverages, nutrient supplement beverages (ready- to- drink, prepared from mix, and dry mix forms)	5g per 240 ml serving (2.08g per 100g)
	Protein drinks, including supplements and diet beverages (ready- to-drink, prepared from mix, and dry mix forms)	1
Milk chocolate	Milk chocolate candies and coatings/coverings	3
Ready-to-eat cereals	All ready-to-eat cereals	3g per 5-55g serving (5-20g per 100g)
Smoothies	Fruit and dairy "smoothie" type beverages	1
Soft/chewy candies	Soft/ chewy candies such as caramels, toffees, taffies, nougats, Creams, fudges, fondant, and fruit- based confectionery (excluding Marshmallows, soft jellies, gummies, panned candies, and liquorice)	3
Chewing gum	Tooth friendly (non-cariogenic) chewing gum	30
Table top sweeteners, low calorie	Sugar substitutes/replacements	1g per serving
Yoghurt	Yoghurt	2

16. The applicant has used dietary survey data to estimate the likely consumption of tagatose in the United States population. These data were taken from the 1994-1996 Continuing Survey of Food Intakes by Individuals (CSFII) on US households and from the 1998 CSFII on children aged 0-9. The data were collected using 24-hour recall interviews for two non-consecutive days and defined according to time and eating occasions. In all cases, it was assumed that all foods or ingredients in each category would contain the NI at the level stated in the table above. A more detailed breakdown and discussion is given in Annex 6 of the dossier. The table below provides a summary of the estimated intake of the NI for US population older than 2 years old:

Summary of the estimated intake of D-tagatose from its proposed food use (excluding chewing gum and food supplements)					
Population	Age	2-day average intake of D-tagatose			
		g/person/day		g/kg bw/day	
		Mean	90 th Percentile	Mean	90 th Percentile
Children	2-5	3.2	6.2	0.19	0.37
Young schoolchildren	6-12	4.3	8.5	0.14	0.28
Teenagers	13-19	4.7	9.5	0.08	0.16
Adults	> 20	4.8	10.5	0.06	0.14
Total population	> 2	4.6	9.8	0.08	0.19

17. The intake of the NI from sugarless chewing gum was based on the results from a separate US survey carried out in 1995. The results of this survey indicate that the average gum consumption in the US population was 2.5 pieces per day. The equivalent figures for pre-school children and teenagers were 1.6 and 3.0 per day. (Annex 6 Table 26).
18. The applicant states that, for technological reasons related to the production of tablets, the intake of the NI via the consumption of food supplements is unlikely to exceed 3g/person/day. The applicant has not explained the derivation of this figure.
19. In response to a request from the Committee, the Secretariat compared the data obtained from the US dietary survey data with the UK NDNS data. The results, calculated using the closest matching food categories are detailed below. These data show comparable levels of consumption would be seen in the UK population.

Comparison of intake estimates based on US and UK dietary survey data					
US Data (g/person/d)			UK data (g/person/d)		
Age Group	Mean	90 th %ile	Age Group	Mean	90(97.5) th %ile
Pre-school (2-5 years)	3.2	6.2	Pre-School (1½ - 4½ years)	2.8	6.9 (10.3)
School Children (6-12 years)	4.3	8.5	School Children (4-18 years)	5.6	11.9 (17.7)
Teenagers (13-19 years)	4.7	9.5			
Adults (> 20)	4.8	10.5	Adults (18- 64)	3.7	9.7 (11.6)

Discussion Estimates of D-tagatose intake for the US and British populations are similar, based on the list of expected uses provided by the applicant. Members noted that higher levels of intake could result in future if the range of uses was expanded or if D-tagatose is incorporated at higher levels.

XI. Nutritional information on the novel food

Information on this aspect is provided on pp 28-34 of the application dossier

20.Reduced Energy Value. Studies described by the applicant indicate that D-tagatose is incompletely absorbed and therefore has a lower energy value compared with sucrose. The applicant refers a number of studies that indicate that the NI has an energy value of 1.5kcal/g. This figure is significantly lower than the value of 4kcal/g that currently applies for the labelling all sugars as specified in the Nutritional Labelling Directive (90/496/EC).

21.Lower glycaemic impact and prebiotic activity. A number of studies were described by the applicant in the dossier. These do not have any bearing on the safety assessment of the novel ingredient.

***Discussion** Members agreed that the studies provided by the applicant in relation to the efficacy of the novel ingredient were not relevant to the safety assessment. It was noted that current European Community nutrition labelling rules require that sugars are labelled to indicate that they supply 4 kcalories/g. A more appropriate value can only be applied for D-tagatose if the applicant seeks an amendment to the Nutrition Labelling Directive (90/496/EEC).*

XII. Microbiological information on the novel food

Information on this aspect is provided in Annex 4 of the application dossier

22. The production of the NI does not involve the use of micro-organisms. The microbiological purity of D-tagatose is detailed in tables 1 and 2 of Annex 4. These data indicate that the final product is essentially free from microbial contamination

***Discussion** Members agreed that the production does not involve the use of a micro-organism and were content that the production process employed by the applicant does not give rise to concern.*

XIII. Toxicological information on the novel food

Information on this aspect is provided on pp p 44-111 of the application dossier

23.Biochemical Aspects (Absorption, distribution and excretion)

The applicant presents a number of studies that indicate a variable and incomplete absorption of D-tagatose. One study also details a pronounced increase in the short chain fatty acids in the blood. SCFA's are produced by bacterial fermentation of the unabsorbed NI in the large intestine. The applicant refers to this 'prebiotic' effect as a tangible benefit that can be attributed to the consumption of the NI.

24. Several studies carried out on humans indicate that intestinal side effects, including stool softening, may occur in susceptible individuals after the consumption of more than 15g D-tagatose (ingested in a single sitting). The

tolerable daily dose is a multiple of the tolerable single dose as the intestinal effects are not cumulative over time.

25. Metabolism

The applicant has referred to a number of scientific studies that demonstrate that the metabolism of D-tagatose takes place along well defined biochemical pathways. Following an initial phosphorylation step, the metabolism converges with the pathway seen for fructose.

26. Toxicological studies

The applicant includes reports from a number of animal studies, which are listed below. The applicant has also conducted four studies indicating a lack of genotoxicity. These studies have also been reviewed by JECFA, which considered D-tagatose three times during 2001-2004. The initial JECFA evaluation of D-tagatose highlighted a number of questions concerning, glycogen deposition and hypertrophy in the liver, and increased serum levels of uric acid.

27. The applicant commissioned a number of additional studies that paid particular attention to these parameters, and following a detailed evaluation JECFA allocated an ADI "not specified" for D-tagatose at its 63rd Meeting in June 2004. The applicant has submitted the same data for novel food approval.

Genotoxicity studies				
Test	Test system	Concentration	Results	Reference
Bacterial gene mutation ^a	<i>S. typhimurium</i> (TA 1535, TA 1537, TA1538, TA98, TA100); <i>E. coli</i> (WP2uvrA)	100-5000 mg/plate	Negative	Lawlor, 1993; Kruger, 1999a
Chromosomal aberration ^{a, b}	Chinese hamster ovary cells	1250-5000 mg/ml	Negative	Murli, 1994a; Kruger et al., 1999a
Micronucleus formation ^d	CD-1 mouse bone marrow	1250-5000 mg/bw (p.o.)	Negative	Murli, 1994a; Kruger et al., 1999a
TK-locus mutation ^{a, c}			Negative	

a) With and without exogenic metabolic activation (rat liver S9 fraction).

b) Treatment time, 7.4h (without activation), 2h (with activation); harvest time 10h

c) Treatment time, 4h

d) Termination 24, 28 and 72h after dosing

Animal studies					
Type of study	Species (N)	Dose level (% of diet or g/kg bw)	Results	NOAEL (% of diet and/or g/kg bw/d)	References
acute toxicity test	Rats (5M, 5F) Mice (5M)	10g/kg bw (single dose)	no mortality or reaction to treatment	10g/kg bw	Trimmer, 1989
Subchronic (90-d) toxicity study	S-D rats (20M 20F / group)	0,5,10,20% 10% fru + 10% cellulose	soft stool (day 1-3); reduced weight gain in 20% group; increased abs. and rel. liver weights in 10, 15, 20% tag groups, some hypertrophy of hepatocytes in 15, 20% group ^a	5% ^{c)} [3.7 (F) and 4.1 (F) g/kg bw/d]	Trimmer et al., 1993 Kruger et al., 1999c
Subchronic (29-31 d) study on liver parameters ^d	S-D rats (20M / group)	0,5,10,20% tag	Dose dependent increase of liver glycogen and lower weight ^{b)} . No ultrastructural (EM) changes of liver tissue except increased glycogen deposition. Slight increased ALAT, ASAT in 20% tag group probably in response	n.d ^{d)}	Lina et al., 1998 Bar et al., 1999
Subchronic (6-month) toxicity study	Wistar rats (60 F/group)	0, 5, 10% tag, 20% fru, 10% tag + 10% fru Interim kills on day 3, 7, 14, 28, 94, 128 (10F / group)	Only liver and plasma parameters were examined. No increase of liver weight and no histopathological changes ^{a)} ; no changes of plasma parameters.	10% of diet [5.8 g/kg bw/d (day 1-28); 4.8 g/kg bw/d (day 1-28)]	Lina & de Bie, 2000d
Chronic (24-month) toxicity/carcinogenicity study	Wistar rats	0, 2.5, 5, 10% tag, 20% fru, 10% tag + 10% fru	Examination of organ weights and his topathology limited to liver, kidneys, adrenals and tests (cecum: weight only). Liver enlargement in 10% tag (M), 20% Fru (M), 10% tag +fru (M&F) but no morphological changes. Increased nephrocalcinosis in females of all tag dose groups and in 10% tag (M) and 10% + 10% fru (M). increased incidence of adrenomedullary proliferative	2.5% of diet [< 1 g/kg bw/d]	Lina & Kuper, 2002 Lina & Bar, 2003

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			disease in 2.5% tag (M), 5% tag (M & F), 10% (M & F) and 10% + 10% fru (M&F)		
Energy balance study (33-d)	Pigs (2 / group)	0, 20% tag, 20% suc, 10% tag + 10 % suc	No ultrastructural (EM) changes of liver tissues	5 g/kg bw/d	Mann, 1997
Embryotoxicity / teratogenicity study (range finding)	S-D rats (5M / group)	0, 4, 8, 12, 16, 20 g tag/kg bw/d (day 6-15 of gestation)	Soft stool and diarrhoea at 12 g/kg bw. (No adverse effect otherwise).	20 g/kg bw/d (11 g/kg bw/d)	Schroeder, 1994a
Embryotoxicity / teratogenicity study	S-D rats (24M / group)	0, 4, 12, 20 g tag/kg bw/d (day 6-15 of gestation)	Maternal liver weight increased in 12 and 20 g/kg bw group. No morphological changes in liver. No adverse effects otherwise.	20 g/kg bw/d	Schroeder, 1994b; Kruger et al., 1999b

Key: M = Male, F = Female

Abbreviations tag, D- tagatose; fru, fructose; suc, sucrose, ALAT, alanine aminotransferase; ASAT, aspartate minotransferase, S-D, Sprague-Dawley, n.d., not determined, bw, body weight.

- a) Animals killed after overnight fasting
- b) Animals killed in the fed condition
- c) Based on effects on liver weight
- d) Liver weight cannot be used as a basis for determination of the NOAEL since rats were killed in the fed condition (increased weight is partly due to liver glycogen accumulation). D- Tagatose intake was about 11.4 g/kg bw/d at the high-dose level.
- e) A series of additional studies on the effects of D- tagatose on liver weight and glycogen accumulation was performed but their results are not shown in this table because toxicological end- points (e.g., histopathology) were not examined.

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Discussion *The novel ingredient has been subject to a number of toxicological studies. The Committee noted the toxicological assessment by JECFA in 2004 and agreed with the expert group that the data did not highlight any toxicologically significant findings, and exhibited properties that were similar to other carbohydrates of other low digestibility.*

Allergenicity and Labelling

Information on this aspect is provided in p 109-110 and Annex 4 of the application dossier

28. The NI is manufactured from crystalline lactose, obtained from cheese whey, which contains protein at levels of up to 0.2%. Recognising the known allergenic potential of milk and derived products, the applicant has demonstrated the absence of whey protein in the NI using an ELISA method. (<10µg protein equivalent / g NI, see Annex 4). The same assay detected protein in 2 (of 3) lactose samples tested.
29. The applicant speculates that the absence of whey protein is to be expected due to the production process, which involves the use of heat-treatment, high pH, ion-exchange resins and activated carbons.
30. In their consideration of the product JECFA concluded that ingestion of 30g or more of the NI may cause gastrointestinal effects in humans. The applicant has also suggested that no warning on laxative effects is necessary for foods listed in the table containing D-tagatose because the maximum intake of D-tagatose would be extremely unlikely to exceed 10g per eating occasion for consumers of any age group (see Table 3 of application dossier). This statement is based on high level US consumption data using figures at the 90th percentile. Estimates using UK NDNS data are similar. The applicant has also acknowledged that the products described in the table are indicative of intended use only, and it would be appropriate to label any foods containing more than 15g of D-tagatose per serving with the statement "excessive consumption may produce laxative effects". This text is in line with the current requirement for polyols (Directive 96/21/EC) which applies to foods containing more than 10% polyols. The applicant's proposal will cover all food categories and is based on the intolerance being induced by the amount, rather than concentration. Unlike polyols, tagatose is proposed for certain beverages, where higher levels of intake may be achieved at a lower concentration of D-tagatose.
31. Following a specific request by the Committee, the applicant submitted additional data to demonstrate that the proposed labelling described above was equally applicable to children as well as adults.

Discussion *Members noted that although the applicant provides evidence that the NI is unlikely to contain whey proteins, the product is derived from a milk source. A new amendment (2003/89/EC) to the food labelling directive (2000/13/EC) requires specified food allergens and their derived ingredients to be included in ingredients listing. Milk is a specified allergen and this requirement therefore applies to the novel ingredient, irrespective of the manufacturing process, unless the applicant applies to the Commission for a formal exemption. Members wished to note that it was their*

view that the data provided to demonstrate that the product was free from milk proteins was unlikely to offer sufficient grounds to qualify for an exemption.

Concerning the potential for exerting a laxative effect, the Committee noted the proposal for labelling on the basis of consumption of more than 15g of the NI in a single serving, similar to the labelling requirement for polyols set out in Directive 96/21/EC. There are no data on the effects of tagatose consumption amongst children although young children are known to be generally more prone to diarrhoea, probably because they have a less developed GI tract. The limited data available on other poorly absorbed compounds, such as sorbitol, indicate that pre-school children may be more sensitive than adults and older children. The applicant does not intend the ingredient to be used in foods specially manufactured for young children but it is likely that they will consume general foods that contain D-tagatose, particularly soft drinks. The Committee therefore considered that the labelling criterion proposed by the applicant is appropriate for solid foods, but proposed that all beverages containing more than 1% D-tagatose should also carry the same advisory labelling.

General discussion

32. Members noted that D-tagatose has been subjected to thorough toxicological testing and agreed with the conclusion of JECFA that it is a substance of low toxicity and does not represent a hazard to health.

33. Like other poorly absorbed compounds, D-tagatose may cause mild gastrointestinal effects in high level consumers. The individual doses of D-tagatose associated with these effects is in the range 15-30 grams which is unlikely to be achieved from consumption of the tagatose-containing foods described by the applicant. Nevertheless, the range of uses may be extended in future and Members supported the applicant's proposal to include advisory labelling on any food product that contained in excess of 15g D-tagatose per serving as being adequate to ensure that consumers were advised of the effect of potential gastrointestinal intolerance. To take account of consumption by young children, and because of evidence that poorly-absorbed compounds may exert a greater laxative effect when taken in liquid form, this advisory labelling should also be applied to all beverages containing more than 1% D-tagatose.

34. Members also noted that allergen labelling as defined in amendment 2003/89/EC to the food labelling directive (2000/13/EC) will apply to all products that contain the NI, unless the applicant applies to the Commission for a specific exemption to be incorporated into the relevant directive.

Conclusion

35. The Advisory Committee on Novel Foods and Processes is satisfied by the evidence provided by Bioresco on behalf of Arla Foods that D-tagatose is acceptable, subject to the applicant's adherence to the proposed specification and the labelling requirements described above.

9 August 2005

SUBMISSION END

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Reference List for Industry Submission, GRN 000352

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WHO. 2004. Safety evaluation of certain food additives and contaminants prepared for the 61st meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) WHO Food Additives Series 52.

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Bonnette, Richard

From: Susan S Cho [susanscho1@yahoo.com]
Sent: Wednesday, December 08, 2010 11:33 AM
To: Bonnette, Richard; Dinovi, Michael J
Cc: 오승현님
Subject: answers to your questions GRN 351

Dear Sir,

It was nice talking to you on the phone on December 7th. We really appreciate your time. Please find our answers to your questions as follows:

1. Exposure estimate:

Food manufacturers may be interested in tagatose because of its glycemic control effects. There are so many sweeteners which are claimed for glycemic control. Thus, tagatose will be used as a replacement for other types of sweeteners and we do not expect any additive effects of various sweeteners. We estimate that actual usage of tagatose will be less than 10% of the intended use outlined in GRN 351. Assuming that 10% of the product will be used at the maximum levels under the intended use, the estimated mean daily intake level is estimated to be 1.62 g/person/day. The US consumption of all types of sugar alcohols (sorbitol, erythritol, maltitol and xylitol) was estimated at 3.43 g/person/day in 2002, of which sorbitol made up more than 54% of the total sugar alcohol production. Actual US consumption of D-tagatose was estimated to be lower than the figure for any single sugar alcohol.

2. Gastrointestinal effects

Gastrointestinal effects of D-tagatose are related to its incomplete absorption in the small intestine and are not unique to D-tagatose. Consumption of sugar alcohols and certain types of soluble fibers (over 15-20g/day) also results in gastrointestinal symptoms, such as bloating, abdominal pain, and diarrhea. However, these are transient symptoms which disappear in several weeks. People will eventually adapt to a high consumption of non digestible carbohydrates without having any gastrointestinal effects. People in some developing countries consume over 60g of fiber daily without having any gastrointestinal problems. Thus, despite potential side effects, the Institute of Medicine, The National Academy of Sciences, recommends an increased consumption of dietary fiber from the current 14-18g to 25-38g per day.

At the end, we think it is a consumers' choice and a marketing issue instead of a safety issue. While working at a food manufacturer which has marketed high soluble fiber products which cause gastrointestinal symptoms in some population, we learned that a majority of people in Western countries tended to stop eating foods causing gastrointestinal effects if they do not want to go through a transient symptom period. People know health benefits, but they may not want to be in a socially embarrassed situation. Only some health conscious persons will go through a transient period.

I hope these answers your questions. If you have any further questions, please contact me. We would really appreciate your kind attention on these matters.

Sincerely,

Susan Cho, Ph.D.
NutraSource
6309 Morning Dew Ct.
Clarksville, MD 21029
410-531-3336

Bonnette, Richard

From: Susan S Cho [susanscho1@yahoo.com]
Sent: Wednesday, December 08, 2010 11:33 AM
To: Bonnette, Richard; Dinovi, Michael J
Cc: 오승현님
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Susan Cho, Ph.D.
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Clarksville, MD 21029
410-531-3336



December 29, 2010

Dr. Richard Bonnette
Division of Biotechnology and GRAS Notice Review
Office of Food Additive Safety-CFSAN
U.S. Food and Drug Administration
5100 Paint Branch Parkway (HFS-255)
College Park, MD 20740-3835

Re: Amendment to GRAS exemption claim for D-tagatose as an ingredient in foods (GRN 351)

Dear Dr. Bonnette,

This is to notify you that CJ Cheiljedang (based in S. Korea) claims that the use of the substance described below (D-tagatose) is exempt from the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act because CJ America has determined such use to be Generally Recognized As Safe (GRAS). We also notify you that CJ Cheiljedang has amended the claim by adding specifications as shown below.

On behalf of CJ Cheiljedang, NutraSource (an independent consulting firm) assembled a panel of experts highly qualified by scientific training and experience to evaluate the safety of the intended uses of D-tagatose. The panel included Dr. Susan Cho at NutraSource (Clarksville, MD), Dr. James Anderson (University of Kentucky, Emeritus, Lexington, KY), and Dr. George Fahey at the University of Illinois (Urbana, IL). Following independent critical evaluation of the available data and information, the panel has determined that the use of D-tagatose (that is manufactured by CJ CheilJedang, S. Korea) described in the enclosed notification is GRAS based on scientific procedures.

After reviewing the available data, the Expert Panel also concluded in its July 2010 statement that the intended use of CJ Cheiljedang's D-tagatose (to be used as an ingredient in foods ready-to eat breakfast cereals, diet soft drinks, non-diet soft drinks, confectionery, formula diets for meal replacement, meal replacement drink mix (powder), cake, pie, cake mix powder, frostings, ice cream and frozen yogurt, yogurt, frostings, sugar free chewing gum, jelly and pudding, coffee mix powder, biscuits, cookies, and cereal bars), resulting in an estimated daily mean intake of 16.2 g D-tagatose and 90th percentile daily intake of 38.9 g, is safe and GRAS for the general population. This determination and notification are in compliance with proposed Sec. 170.36 of Part 21 of the Code of Federal Regulations (21 CFR section 170.36) as published in the Federal Register, Vol. 62, No. 74, FR 18937, April 17, 1997.

Notifier's name and Address: CJ Cheiljedang, Inc.
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Name of GRAS substance: D-Tagatose (Common or trade name: Tagatose or pseudo-fructose).

Product description: D-Tagatose is a ketohexose, an epimer of D-fructose isomerized at C-4. D-Tagatose differs from fructose only in the positioning of the hydroxyl group on the fourth carbon. D-Tagatose is 92% as sweet as sucrose. Functional uses of D-tagatose as food additives are as a sweetener, texturizer, stabilizer, humectant, and formulation aid. D-Tagatose provides several health benefits to consumers: 1) it does not promote tooth decay, 2) it provides approximately 1.5 kcal/g to the diet, and 3) it attenuates a glycemic response. D-Tagatose has a history of use in foods with no reported adverse effects.

Specifications:

Table 1. Specifications of D-tagatose

Parameter	Specification
D-Tagatose	>98.0% (wt/wt)
D-Galactose and other sugars	≤ 1.5% (wt/wt)
Moisture	≤0.5 % (wt/wt)
Ash	≤ 0.1% (wt/wt)
Total plate count	<10,000 CFU/g
Coliforms	negative
<i>Staphylococcus aureus</i>	negative
Hg	<0.1 ppm
Cd	<0.1 ppm
Lead	<0.5 ppm
As	<1.0 ppm
Physical Appearance	White crystal

Applicable conditions of use of the notified substance

D-Tagatose is expected to be used in ready-to-eat breakfast cereals, diet soft drinks, non-diet soft drinks, confectionery, formula diets for meal replacement, meal replacement drink mix (powder), hard and soft confectioneries, cake, pie, cake mix powder, frostings, ice cream and frozen yogurt, yogurt, frostings, chewing gum (sugar free), jelly and pudding, coffee mix powder, biscuits, cookies, and cereal bars. The proposed use levels of D-tagatose are presented in Table 2.

Table 2. Proposed food application of D-tagatose and maximum levels of use

Food category	Maximum level
Ready-to-eat breakfast cereals	10 g/30 g
Diet soft drinks	5 g/240 ml
Non-diet soft drinks	7.5 g/240 ml
Confectionery	7.5 g/30 g
Formula diets for meal replacement	7.5 g/240 ml
Meal replacement drink mix (powder)	2 g/6 g
Cake, pie,	10 g/100 g
Cake mix powder	15 g/100 g
Frostings	15 g/100 g
Ice cream and frozen yogurt	7.5 g/100 g
Yogurt	7.5 g/100 g

Chewing gum, sugar free	60%
Jelly and pudding	7.5 g/30 g
Coffee mix powder	7.5 g/20 g
Biscuits	10 g/100 g
Cookies	10 g/100 g
Cereal bars	10 g/40 g

Exposure estimates

If 100% of the products are used at the maximum levels, the mean intakes including D-tagatose from all GRAS proposed use categories by users of one or more foods is 16.2 g/d for the American population aged 1 and above. The 90th percentile intakes including D-tagatose from all GRAS proposed use categories by users of one or more foods are 38.9 g/d for the population combining males and females, 44.9 g/d for males and 32.4 g/d for females. These levels correspond to 695, 773, and 620 mg/kg BW/d for the entire population, males, and females aged 1 and over. These levels are much lower than the no-observed-adverse-effect level (NOAEL) values (up to 6,700 mg/kg BW/d) that have been found from sub-chronic and chronic toxicity studies in rats. Also, these estimated daily exposure levels are below the an upper safe level of 45 g/d that has been established by WHO (Boesch et al., 2001; WHO, 2004).

Basis of GRAS determination

Through scientific procedures.

Review and copying statement

The data and information that serve as the basis for this GRAS determination will be sent to the FDA upon request, or are available for the FDA's review and copying at reasonable times at the office of CJ Cheiljedang, Inc. or Nutrasource, Inc.

We enclose an original and two copies of this notification for your review. If you have any questions, please contact me.

Sincerely,

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