



Subchronic toxicity, mutagenicity and allergenicity studies of a cultured dextrose food product

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Abstract

MicroGARD[®] 200 is a fermented dextrose product used to extend food shelf-life by inhibiting spoilage due to Gram-negative bacteria, selected yeast and molds. The present studies were conducted to evaluate the safety of this food ingredient for determination of GRAS status. MicroGARD 200 was subjected to a bacterial reverse mutation assay, a subchronic oral toxicity study and an oral antigenicity study. It showed no evidence of mutagenic potential or toxicity in four strains of *Salmonella typhimurium* or in *Escherichia coli* strain WP2 *uvrA* with and without metabolic activation. MicroGARD 200 was orally administered to rats for 13 consecutive weeks at dietary concentrations of 0, 0.5, 2.0 or 5.0%. Water consumption and urinary excretion of sodium were slightly increased in both sexes at the high dose due to the sodium content of the test substance (about 6%). Increases in fasting glucose and decreased plasma creatinine were not accompanied by treatment-related histopathological changes and were within the normal range for historical controls. The potential antigenic properties of MicroGARD 200 were investigated via gavage in guinea pigs using an active systemic anaphylaxis (ASA) challenge [Annals of Allergy 67 (1991) 400; Allergy 49 (1994) 361]. There was no evidence of any anaphylactic sensitizing properties for MicroGARD 200.

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1. Introduction

MicroGARD[®] 200 is a cultured dextrose powder that has been used commercially since 1988 as an alternative to chemical preservatives to retard spoilage in a variety of foods. This non-dairy dextrose fermentation product enhances flavor, retains packaged freshness and extends shelf-life because it inhibits spoilage due to Gram negative psychrotrophic bacteria and selected yeast and molds. Fig. 1 presents viable colony count determinations for a common organism of putrefaction, *Pseudomonas putida*, when incubated in various concentrations of MicroGARD 200.

Fig. 2 presents data on the zones of inhibition for several common yeast, including *Saccharomyces cerevisiae*,

which has a long history of use in commercial fermentation. In this experiment, 5-mm diameter wells were cut into yeast seeded agar, filled with approximately 50 µl of water resuspended MicroGARD 200 and allowed to incubate overnight. Inhibitors present in MicroGARD diffuse into the agar surrounding the well and can inhibit the outgrowth of sensitive organisms. As a result of this inhibition, clear zones of no growth around the well (measured in millimeters) are seen. The zone width represents a relative indication of the sensitivity of each particular species of yeast to MicroGARD 200. In the absence of MicroGARD (controls), there is no yeast inhibition and consequently no clear zone surrounding the well.

MicroGARD 200 is also effective against a variety of food molds such as *Rhizopus nigricans*, which is a common bread mold (data not shown).

Mutagenicity, subchronic toxicity and allergenicity studies were conducted in order to support the determination of the GRAS status of MicroGARD 200. All experimental work was conducted by TNO Nutrition

Abbreviations: ALP, alkaline phosphatase; ASA, active systemic anaphylaxis.

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and Food Research Institute (Zeist, The Netherlands) in compliance with OECD Principles of Good Laboratory Practice (OECD, 1998).

2. Materials and methods

2.1. Test substance

The test substance for each study was MicroGARD 200, which is manufactured by controlled fermentation of dextrose with *Propionibacteria freudenreichii* subsp. *shermanii*. This off-white powder was obtained from the manufacturer (Rhodia). Analysis of the composition revealed that the test substance was approximately 72% maltodextrin, with the balance being fermented dextrose by-products including yeast extract, organic acids and uncharacterized metabolites. Additional analysis of

MicroGARD 200 for sodium found 6.1 g/100 g. Batch Number 200-9316AA was used for the allergenicity study while batch number A7-343 RED was utilized for the remaining studies.

2.2. Bacterial reverse mutation test with MicroGARD 200

This assay is based on the work of Ames et al. and was conducted in accordance with applicable OECD, EEC and US EPA Health Effects Testing Guidelines (Ames et al., 1975; Maron and Ames, 1983; US EPA, 1989; OECD, 1997; EEC, 1992). MicroGARD 200 was subjected to mutagenicity testing in the bacterial reverse mutation assay using histidine-requiring *Salmonella typhimurium* strains TA1535, TA1537, TA98 and TA100 and the tryptophan-requiring *Escherichia coli* strain WP2 *uvrA*. No preliminary screening for toxicity

Colony Forming Units

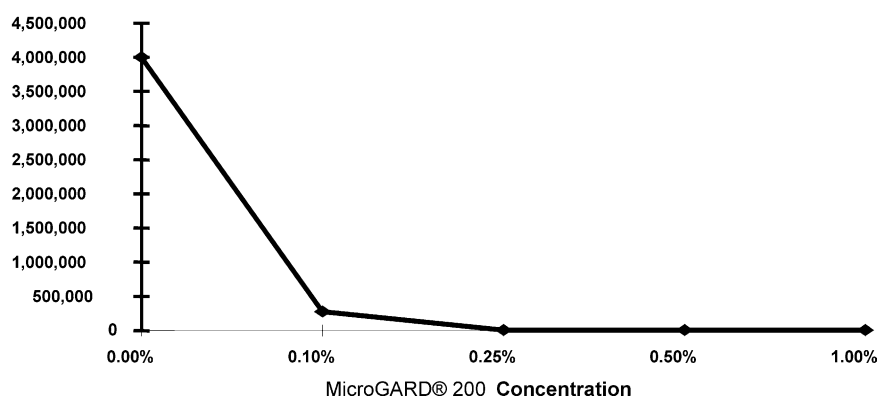


Fig. 1. Efficacy of MicroGARD 200 against *Pseudomonas putida*.

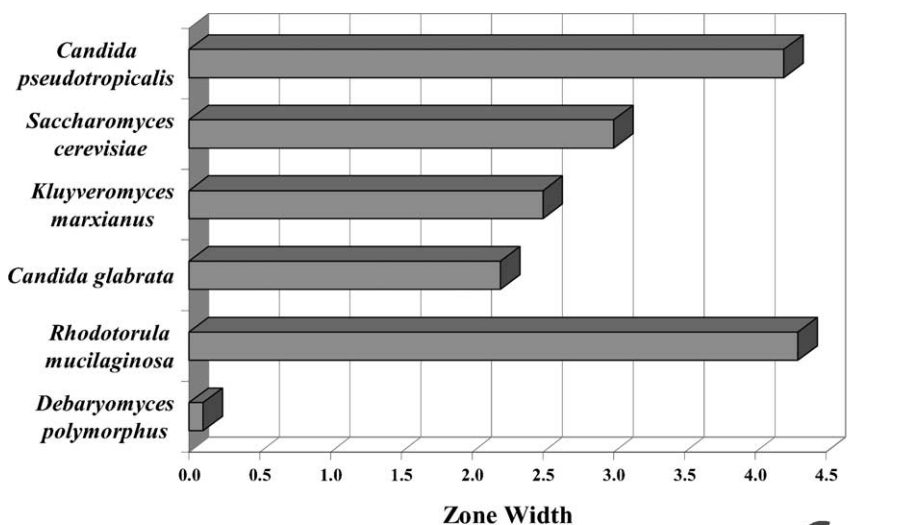


Fig. 2. Efficacy of MicroGARD 200 for inhibition of common yeast.

was performed because it was not expected. Instead, the toxicity test was incorporated into the assay.

Water was used as the vehicle for the test substance and serial dilutions were made so that six different concentrations of MicroGARD 200 ranging from 62 to 5000 µg/plate were assayed. Negative and positive controls were evaluated simultaneously. All determinations were made in triplicate. The batch of S9 used for exogenous metabolic activation was prepared prior to testing using 12 Wistar rats injected intraperitoneally with a single dose of Aroclor 1254 and met all in-house quality criteria.

2.3. Subchronic (13-week) oral toxicity study in rats

Four groups of 20 male and 20 female Wistar rats were administered dietary concentrations of 0, 0.5, 2.0 or 5.0% MicroGARD 200 on a daily basis for 13 consecutive weeks. Parameters evaluated in this study included clinical observations (am and pm on workdays), body weight, food/water consumption, food conversion efficiency, ophthalmology, hematology, clinical chemistry, urinalysis, absolute and relative organ weights and gross and histopathological examination of tissue. The study protocol was designed to be consistent with guidelines from the US Food and Drug Administration, OECD and EEC (OECD, 1981; US FDA, 1982; EEC, 1988).

Rats were approximately 4 weeks of age at study initiation and the mean weights ($\pm$ S.D.) were 133.4 ± 1.8 g for males and 112.3 ± 1.2 g for females. Animals were assigned to treatment groups via a computerized randomization program based on body weight, and individually identified by ear tattoo. From arrival until the last week of treatment, animals were housed five per cage, separated by sex, in suspended stainless-steel cages. In the final week of treatment, animals were separated and housed individually for 24 h in stainless-steel metabolism cages to collect urine for analysis. A 12-h light/dark cycle was observed and there were approximately 10 air changes per h in the room. Although some fluctuation occurred in relative humidity due to room cleaning, humidity generally ranged from 30 to 70% and temperatures were maintained at 19–24.5 °C. Food and water were provided ad lib. unless precluded by clinical chemistry or hematology evaluations. The test substance was mixed with commercial rodent diet (Rat & Mouse No. 3 Breeding Diet, RM3, Witham, UK), which had been assayed by the supplier for nutrients and contaminants. Fresh batches of experimental diets were prepared at test concentrations of 0, 0.5, 2.0 or 5.0% on three different occasions during the study using a mechanical blender and stored frozen between uses. Appropriate GC methods were used to verify stability, homogeneity and concentration of the test substance in the diet used in this study.

Clinical observations were conducted twice per day on workdays and once per day on weekends and holidays. Body weights were recorded pre-study, day 0 (day of dose initiation) and weekly thereafter and on the day of sacrifice. Food consumption was measured per cage over successive 1-week periods and food conversion efficiency was calculated. Water consumption was measured in animals slated for urine collection during 5-day periods in weeks 1, 6 and 12 and at week 13. Control and high-dose animals were subjected to ophthalmoscopic examination 5 days prior to the start of the study and at day 86 after induction of mydriasis using an atropine sulfate solution. Ten rats per sex per group were subjected to hematological evaluations and clinical chemistry at necropsy, using blood collected via abdominal aorta. Table 1 presents the hematology, clinical chemistry and urinalysis parameters determined in this study.

Gross necropsy and organ weights were obtained on days 91–92 (10 males/group/day) and on days 93–94 (10 females/group/day). Exsanguination from the abdominal aorta was performed under anesthesia. Organ weights were obtained for the following organs: adrenals, brain, caecum (full and empty), heart, kidneys, liver, spleen, testes, thymus and thyroid (with parathyroids). Table 2 presents the tissues taken at necropsy and those weighed at that time.

Tissues taken at necropsy were examined grossly for pathological changes and preserved in an aqueous phosphate buffered 4% solution of formaldehyde. All tissues were then embedded in paraffin wax, sectioned at 5 µm, stained with hematoxylin and eosin and examined microscopically with the exception of the axillary lymph nodes, exorbital lachrymal glands, femur with joint, mandibular lymph nodes, parotid salivary glands and skin. These tissues were only examined if a change was observed on gross examination.

Statistical analysis of the data was conducted using two-sided tests and probability values of $P < 0.05$ were considered significant. Histopathology changes were evaluated using Fisher's exact test. Relative differential white blood cell counts and urinary parameters except for volume and density were subjected to Kruskal–Wallis non-parametric ANOVA followed by Mann–Whitney *U*-tests. Other parameters were generally evaluated using one-way ANOVA followed by Dunnett's multiple comparison tests. Independent of the ANOVA results, homogeneity of variances were tested using Bartlett's test. If variances were determined to vary significantly ($P < 0.01$), then the Kruskal–Wallis nonparametric ANOVA was performed. While statistical significance was considered an important tool for study interpretation, the final determination of the importance of any given statistically significant finding was based on biological, pathological and dose-response factors.

Table 1
Hematology, clinical chemistry and urinalysis

<i>Hematology (10 rats per sex per group at study termination)</i>			
Hemoglobin concentration	Packed cell volume	Red blood cell count	Reticulocyte count
Total leukocyte count	Differential leukocyte count	Prothrombin time	Thrombocyte count
Mean corpuscular volume	Mean corpuscular hemoglobin	Mean corpuscular hemoglobin concentration	Blood smears
<i>Clinical chemistry (10 rats per sex per group (same as for hematology) at study termination)</i>			
Alkaline phosphatase activity (ALP)	Aspartate aminotransferase activity (ASAT)	Alanine aminotransferase activity (ALAT)	Gamma glutamyl transferase activity (GGT)
Total protein	Albumin	Albumin/globulin ratio	Urea
Creatinine	Bilirubin (total)	Cholesterol (total)	Triglycerides
Phospholipids	Inorganic phosphate	Calcium (Ca)	Chloride (Cl)
Potassium (K)	Sodium (Na)		
<i>Urinalysis (conducted on the same rats as above at days 87–88)</i>			
Volume	Density	Appearance	pH
Glucose	Occult blood	Ketones	Protein
Bilirubin	Urobilinogen	Sodium electrolytes	Potassium electrolytes
Chloride electrolytes	RBCs/WBCs in sediment	Epithelial cells in sediment	Amorphous material (sed.)
Crystals in sediment	Casts in sediment	Bacteria in sediment	Sperm cells in sediment
Worm eggs in sediment			

Table 2
Histopathological evaluation of tissues (organ weights were also taken^a)

<i>Tissues taken at necropsy (organ weights were also taken^a)</i>			
Adrenals ^a	Aorta	Brain (stem, cerebrum, cerebellum) ^a	Caecum ^a
Colon	Epididymides	Exorbital lachrymal glands	Eyes
Femur with joint	GALT (gut associated lymphoid tissue) including Peyer's patches)	Heart ^a	Kidneys ^a
Liver ^a	Lungs	Lymph nodes (axillary, mandibular [cervical], mesenteric)	Mammary gland (females only)
Sciatic peripheral nerve	Esophagus	Ovaries	Pancreas
Parathyroids ^a with thyroid	Pituitary	Prostate	Rectum
Seminal vesicles with coagulating glands	Skeletal muscle (thigh)	Skin	Small intestine (duodenum, ileum, jejunum)
Spinal cord (three levels)	Spleen ^a	Sternum with bone marrow	Stomach (glandular and non-glandular)
Salivary glands (parotid, sublingual, submaxillary)	Testes ^a	Thymus ^a	Thyroid ^a
Trachea/bronchi	Urinary bladder	Uterus with cervix	Any gross lesions

2.4. Determination of active systemic anaphylaxis in guinea pigs

The possible antigenic potential of MicroGARD 200 (and any bacterial protein it may contain) was determined after oral administration by gavage in male guinea pigs using active systemic anaphylaxis (ASA) challenge according to the methods described by Piacentini et al. (1991, 1994). Analysis of the test substance for identity, quality and purity was performed by Rhodia, Inc., but protein content of MicroGARD 200 was confirmed by the testing facility.

Fifty male guinea pigs (including 14 reserves), approximately 8 weeks of age, were received from

Charles River Deutschland (Sulzfeld, Germany). Animals were identified by ear tattoo, acclimatized for 9 days and housed in a mobile battery containing six cages with six animals per cage. Temperature was maintained at 22.4 ± 0.3 °C and relative humidity ranged from 47 to 65%. Approximately 10 air changes occurred per h in the animal room. Food and water were available ad lib. and consisted of Guinea Pig Diet FD1 (Witham, UK). Both food and water had been assayed previously for contaminants. Routine serology was performed prior to the start of the study and animals were assigned to groups proportionally based on body weight. Clinical observations were performed daily and body weights were determined at day 0 and then weekly until

Table 3
Study design

Group/subgroup	No. of animals (all males)	Sensitization		ASA challenge	
		Solution	Oral dose per day per animal (days 0–36)	Solution	iv dosing per animal at day 49
A subgroup 1	3	Water for injection	1.5 ml	MicroGARD 200	7.5 mg protein in 1.5 ml
A subgroup 2	3	Water for injection	1.5 ml	Ovalbumin	5 mg in 1.5 ml
B (test group)	6	MicroGARD 200	1 g (w/v)/ml. Final volume is 1.5 ml (containing 33 mg protein)	MicroGARD 200	7.5 mg protein in 1.5 ml
C (test group)	6	MicroGARD 200	1 g (w/v)/ml. Final volume is 1.5 ml (containing 33 mg protein)	MicroGARD 200	7.5 mg protein in 1.5 ml
D (neg. control)	6	MicroGARD 200 Control	1.5 ml with an amount of protein comparable to group B	Control (without bacteria or fermentation)	7.5 mg protein in 1.5 ml
E (neg. control)	6	MicroGARD 200 Control	1.5 ml with an amount of protein comparable to group B	MicroGARD 200 Control (without bacteria or fermentation)	7.5 mg protein in 1.5 ml
F (positive control)	6	Ovalbumin	1 mg in 1.5 ml	Ovalbumin	5 mg in 1.5 ml

the ASA challenge on day 49. On days 0 and 38, blood samples were taken via orbital puncture for optional PCA tests and antibody determinations by ELISA.

This study consisted of six groups (designated A through F) with two test groups and the remainder as controls. MicroGARD 200 CONTROL was a mixture of MicroGARD 200 dry non-dairy ingredients without the addition of bacteria or fermentation while MicroGARD 200 is the product as marketed. Water served as a negative control and ovalbumin as a positive control. Treatment groups were challenged by either the test substance, MicroGARD 200 (fermented product) or by MicroGARD 200 CONTROL, the mixture of starting ingredients without bacteria or fermentation.

Table 3 presents a schematic of the study design and doses.

The ASA test occurred on day 49 and consisted of an intravenous injection into the penile vein of the appropriate challenge dose (see Table 3). Observations occurred at 30 min and then at 3 h for any signs of anaphylaxis, which consisted of piloerection, nose licking or scratching, unrest, tremor, sneezing/coughing, urination/defecation, respiratory difficulties, ataxia, convulsions or death.

3. Results and discussion

3.1. Bacterial reverse mutation test

Two separate mutagenicity assays were performed. With the exception of the second assay for TA1537, without activation, no changes were observed. In the

second assay for TA1537, a two-fold increase in the number of revertant colonies was observed in the absence of metabolic activation when compared with the negative control. Since this finding was not observed in the first assay, it was considered a chance finding and not biologically relevant. A third assay was considered unnecessary based on the lack of increase in revertant colonies in the first assay, no evidence of dose response, mutagenicity or toxicity in any of the other strains or in TA1537 with activation and, finally, unusually low values for the associated negative controls (even though they were within acceptable ranges). Therefore, it was concluded that MicroGARD 200 showed no evidence of cytotoxicity or mutagenic potential in *Salmonella typhimurium* strains TA1535, TA1537, TA98 and TA100 or in the *Escherichia coli* strain WP2 *uvrA* in either the presence or absence of metabolic activation.

3.2. Subchronic (13-week) oral toxicity study in rats

Analysis of the administered diet confirmed target concentrations, homogeneity of the diet mixture and stability of the test substance for the appropriate time period in the diet. No treatment-related changes or adverse effects were observed in any clinical observations, body weight, food consumption or conversion efficiency, ophthalmology, hematology or gross pathology. Test material intake did decrease somewhat with study duration due to the well-known decreases in food consumption that occur as the test species age. Mean intakes of test material were 378, 1513 and 3700 mg/kg/day for males and 405, 1598 and 4100 mg/kg/day for females at dietary concentrations of 0.5, 2.0 and 5.0%, respectively. A slight increase in water consumption,

which was sometimes statistically significant, was noted at the high dose. No consistent changes were observed at the low- and mid-doses. This change is not considered a direct effect of the material or an indication of any toxicity; rather it can be attributed to the high sodium content of the test material, which is approximately 6%. This correlates with elevated excretion of sodium observed in both sexes at the high dose. High-dose rats consumed about twice as much sodium as controls. No other urinalysis findings showed any effects attributable to MicroGARD 200. A few statistically significant differences between treated rats and controls were noted with respect to clinical chemistry. Fasting glucose was increased in high-dose females and high-dose males had decreased creatinine levels. These findings were not accompanied by any treatment-related histopathological changes and were well within the normal range of historical controls at the testing facility. Decreased alkaline phosphatase (ALP) was observed in low- and high-dose groups, but given the lack of a clear dose-response relationship, these findings were considered incidental. There was a statistically significant increase in absolute testes weights at the mid-dose, but this was considered an incidental finding in the absence of a statistically significant increase in the relative testes weight, any evidence of dose response or histopathological changes. Similarly, a statistically significant increase in aggregates of reticulo-endothelial system cells and necrotic hepatocytes in the low-dose females was considered a chance finding due to lack of any dose response.

3.3. Determination of active systemic anaphylaxis in guinea pigs

Two animals were replaced during the study due to gavage errors involving the lungs of the animals originally selected for testing. Body weights in this study increased in the Group F ovalbumin controls; this was probably related to the high protein contained in doses of ovalbumin. There were no clinical observations related to the test material and no animals showed any signs of anaphylaxis except for positive controls, which is expected and demonstrates the sensitivity of the model. Serum samples collected on days 0 and 38 were used to determine whether the lack of ASA results represented the absence of antigenic properties. As expected, positive control ovalbumin serum titers were significantly elevated on day 38 as compared with day 0. Negative control group ovalbumin samples were below the limit of detection. In the remaining samples, five out of 30 samples (one out of six per group) showed small elevations in MicroGARD 200-specific IgG levels when compared with day 0. This was approximately a 16 to 128-fold increase as compared with the ovalbumin control group which showed a greater than 8000-fold increase. However, this was not considered to be an

indication of antigenic potential because in the negative control group, one out of six animals exhibited an increase of approximately 128-fold after a 37-day treatment of water. Additionally, the absence of anaphylactic symptoms on day 49 in the ASA challenge demonstrates that MicroGARD 200 does not have anaphylactic sensitizing properties.

4. Conclusions

Overall, it can be concluded that MicroGARD 200 showed no evidence of mutagenicity, subchronic toxicity or allergenicity in the three tests that were performed. The dietary no- observed-effect level (NOEL) for MicroGARD 200 based on the subchronic study in rats was determined to be 5.0%, the highest concentration tested, which was equivalent to an overall intake of 3.7 and 4.1 g/kg body weight/day in males and females, respectively.

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