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Safety evaluation of an α-amylase enzyme preparation derived from the archaeal order *Thermococcales* as expressed in *Pseudomonas fluorescens* biovar I

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Abstract

BD5088 α-amylase derived from archaeal sources has characteristics of pH and temperature tolerance that are well suited to hydrolysis of starch in food processing applications. The production microorganism recipient strain, *Pseudomonas fluorescens* biovar I, strain MB101, was avirulent after oral administration to mice and does not represent an infectious threat to humans. Repeated dose gavage studies with BD5088 enzyme preparation, up to 13 weeks in duration, showed no systemic toxicity due to the oral route with an NOAEL of 890 mg/kg/day as Total Organic Solids. Some irritation occurred in the respiratory tract, which was considered to be a consequence of reflux and aspiration of test material that contained lipopolysaccharide from the *Pseudomonas* production strain. A 2-week dietary study (0 and 310 mg/kg/day) confirmed that there were no respiratory tract effects related to oral ingestion. There was no genotoxic activity based on Ames, mouse lymphoma, mouse micronucleus, and rat lymphocyte chromosomal aberration tests. There was no evidence of allergenic potential based on a comparison of the primary sequence of BD5088 with sequences in an allergen database. The enzyme was labile to pepsin digestion. Based on these data, BD5088 α-amylase preparation may be considered safe for use in food production such as corn wet milling.

Keywords: CAS 9000-90-2; EINECS 232-565-6; IUB 3.2.1.1; Hyperthermophile; Extremozyme; Alpha-Amylase; Archaea; Enzyme; Safety; Pseudomonas fluorescens

1. Introduction

The safety of microbially derived enzyme preparations is established by information on history of safe use and by scientific studies (IFBC, 1990; Pariza and Johnson, 2001). This article presents an assessment of safety for a specific α-amylase enzyme preparation, produced through intergeneric transfer of genetic material and used in the hydrolysis of starch, with applications in food processing including the production of high-fructose corn syrups, nutritive sweeteners, and potable and power ethanol production. This series of studies establishes the initial toxicological profile for a novel α-amylase enzyme derived from a *Thermococcales* microorganism expressed in *Pseudomonas fluorescens*. The assessment is consistent with the procedures used in safety evaluations of enzyme preparations conducted by the U.S. Food and Drug Administration (FDA, 1983, 1990, 1992a,b), with recommendations of international safety review bodies (JECFA, 1987, 1992, 2001) and by food standards compendia (FCC IV, 1996).

The development and optimization of the BD5088 α-amylase enzyme is described elsewhere (Richardson

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et al., 2002). Microbial DNA libraries were screened to identify α-amylases with characteristics compatible with large-scale corn wet milling process conditions. This screening identified 15 clones as primary hits based upon activity without addition of Ca^{2+}, either at pH 4.5 or at high temperature (95 °C). Three enzyme candidates from the archaeal Order *Thermococcales* were subsequently chosen based on exceptional expression of one or more aspects of the necessary characteristics, i.e., temperature stability, pH optimum, lowered reliance on Ca^{2+}, and/or enzyme rate. Two of the BD5088 α-amylase parents, BD5031 and BD5064, were isolated from *Thermococcus* species harvested from shallow marine hydrothermal systems at 95 °C, pH 7.0 and 85 °C, pH 6.0, respectively. The third α-amylase parent, BD5063, was isolated from a primary enrichment culture from the Deep Sea Pacific Ocean, temperature 90 °C and pH 6.5. BD5063 was a *Pyrococcus* or *Thermococcus* species, based upon DNA sequence comparisons with other archaeal α-amylases.

To combine the desirable aspects of the three parental phenotypes, the natural gene homologues were used as a parental sequence set for gene reassembly. Approximately 21,000 chimeric daughter sequences were generated and subsets screened using a process-specific, high-throughput activity assay. This process yielded numerous improved mutants with combined optimal properties of expression, temperature stability, and pH. Biochemical and process-specific characterization of these gene products identified the BD5088 α-amylase for exceptional process compatibility and economics.

The *Archaea* is one of the three biological domains to which all living organisms belong (Woese et al., 1990). Early reports on the archaea have generally characterized organisms belonging to this domain as living under extreme conditions (e.g., high salt, high temperature, low pH) and inhabiting a limited number of environments or specialized ecological niches. However, recent applications of molecular phylogenetic techniques have shown that archaeal organisms are widespread in nature and comprise a significant fraction of the microbial communities in soil (Bintrim et al., 1997), freshwater sediments (MacGregor et al., 1997), and coastal marine environments (DeLong, 1992). The recently reported ubiquity of the archaea supports that these organisms play a significant role in nature (Stein and Simon, 1996). Archaeal organisms found in extreme environments can serve as a diverse source of proteins with highly unusual and useful characteristics. Although the potential significance of starch-hydrolyzing enzymes from thermophilic microorganisms has been recognized (Bertoldo and Antranikan, 2002), BD5088 α-amylase is the first enzyme from this source known to have been developed for commercialization. This enzyme has characteristics of pH and heat tolerance that are well suited for the conditions of starch processing by corn wet milling.

α-Amylases as a class (IUB No. 3.2.1.1) are endo-acting enzymes that hydrolyze α-1,4-glucosidic linkages in amylose and amylopectin to produce soluble dextrins and oligosaccharides. α-Amylases of bacterial and fungal origin have been used in food processing for many years. These include specific α-amylases used to hydrolyze edible starch to produce maltodextrin and nutritive carbohydrate sweeteners that have been affirmed by FDA as being Generally Recognized as Safe (21 CFR, Section 184.1012), as well as other FDA permitted enzymes (e.g., flour may contain α-amylase obtained from the fungus *Aspergillus oryzae*; 21 CFR Section 137.105). As an important new α-amylase enzyme from archaeal origins, the BD5088 preparation was evaluated in a series of studies designed according to current guidance on evaluating the safety of microbial enzyme preparations used in food processing (Pariza and Johnson, 2001). This approach addresses concerns regarding the safety of the production strain with respect to toxigenic and pathogenic potential, as well as the safety of the engineered enzyme itself.

These evaluations are specifically directed at establishing the safety of what will be very low levels of residual enzyme preparation in finished foodstuffs. The final goal of the Pariza and Johnson (2001) decision tree is a sufficiently high No Observed Adverse Effect Level (NOAEL), in an appropriate oral toxicity study, to ensure safety. Therefore, a subchronic study in Fischer 344 rats was conducted by gavage as a well-defined relevant administration route in a commonly used rat strain. In addition, in vitro and in vivo genotoxicity evaluations were conducted to address possible regulatory requirements, as were skin and eye irritation studies for industrial handling guidance purposes. The estimated dietary intake of Total Organic Solids (TOS) derived from the BD5088 α-amylase enzyme preparation, based on worst-case anticipated consumption of corn sweeteners (including high fructose corn syrup (HFCS) and both crystalline and syrup forms of glucose/dextrose) is 3.6 μg/person/day. This value was used in conjunction with the subchronic NOAEL to establish an adequate Margin of Exposure. The toxicology studies were conducted in compliance with FDA regulations for Good Laboratory Practice for Nonclinical Laboratory Studies (21 CFR Part 58), OECD guidelines (ENV/MC/CHEM(98)17) and in accordance with current FDA test guidelines (FDA, 1982; FDA, 2000).

Allergy in bakery workers has been associated with a protein component of a fungal (*A. oryzae*) amylase preparation (Baur et al., 1994; Quirce et al., 1992). Since allergenic responses have been associated with airborne exposure to protein-derived materials, the Enzyme Technical Association (2000) has provided useful guidance on the industrial handling of enzymes in order to control airborne exposure. This handling guidance has been followed for BD5088 as a precaution. The work in

this paper, however, is directed at evaluating safety in food, and not towards occupational exposure.

Bioinformatic searches and pepsin digestibility studies were conducted to help evaluate the potential for food allergenicity. A comparison of the amino-acid sequence of BD5088 to known protein allergens (which included food allergens) is one step in a multilevel analytical process to assess allergenic potential (Metcalfe et al., 1996). These tests were used to evaluate whether there is a possibility that an introduced protein shares either a linear or conformational epitope with a known allergen. Evaluation of amino-acid sequence homology of BD5088 included a comparison with TAKA-amylase, an amylase from *A. oryzae* identified in an allergen database.

2. Materials and methods

2.1. Test material preparation and characterization

The enzyme production process was based on procedures commonly used in fed-batch fermentation of recombinant microorganisms, where fermentation is followed by enzyme recovery (microfiltration), concentration (ultrafiltration), and formulation. A DNA removal step was included to eliminate residual recombinant DNA from the final preparation. Production of the enzyme follows good manufacturing practices (GMP) for food. Raw materials and formulation aids were either food-grade or were equivalent materials commonly employed by the enzyme industry.

Test materials were produced by submerged fermentation of a recombinant *P. fluorescens* biovar I, strain DC88 (the modified production microorganism). The initial recipient or parent strain of the production strain, *P. fluorescens* MB101, was isolated from a Southern California farm lettuce leaf, and has been utilized since 1989 for the large-scale commercial manufacture of *Bacillus thuringiensis* (BT) insecticidal protein preparations for use in agricultural applications. The recipient strain MB101 and the recombinant production strain DC88 have been extensively characterized to the species and biovar level via phenotypic, genomic (16S rRNA gene sequence analysis) and biochemical means. Strain DC88 was derived by inserting stable, non-conjugative and poorly mobilizable plasmids coding for the BD5088 amylase gene into strain MB101. The production strain complies with both the Organization for Economic Co-operation and Development criteria for Good Industrial Large Scale Practice for recombinant microorganisms (OECD, 1992) and the National Institutes of Health Guidelines for Recombinant DNA Molecules (NIH, 1994). Based upon these criteria, a BL1-LS containment level is appropriate for the production strain.

The BD5088 enzyme test material differed from the intended commercial formulation in that it did not contain otherwise safe food preservatives and additives, e.g., NaCl, that might affect animal nutrition or palatability at high levels. However, as an *enzyme preparation*, it contained the component α-amylase and metabolites of the production strain; hence it was truly representative of the process for the final enzyme product. In order to obtain a sufficiently high TOS concentration for toxicology studies, enzyme preparations were lyophilized to a dry powder and reconstituted to the desired concentration as needed. The concentration intended for commercial use is approximately 3% TOS, with the balance consisting of water and food-grade ingredients, where $\%\text{TOS} = 100\% - (\%\text{ash} + \%\text{water} + \%\text{diluents})$.

For the 5-day gavage study, the single lyophilized test batch of BD5088 contained 79% TOS, 16% ash, and 4.5% volatile materials (such as residual water) with an α-amylase enzyme activity of 1.8 activity units/mg dry solids. Amylase activity for all test batches was determined via a continuous spectrophotometric assay, by measuring the release of *p*-nitrophenol from the substrate, 5 mM *p*-nitrophenyl-α-D-hexa-(1,4)-glucopyranoside in 50 mM MOPS buffer, pH 7.0, at 75 °C. One activity unit (AU) was defined as the amount of enzyme required to catalyze the release of 1 μmol/ml/min of *p*-nitrophenol under the defined conditions of the assay. Under industrially relevant conditions, 50,000 amylase units will hydrolyze approximately 1000 kg of starch.

For all other studies, a four-batch composite reference lot of material was used. This composite was made by pooling enzyme preparations from four separate fermentation and recovery runs and lyophilizing as a single batch. The composite batch contained 89% TOS, 9.7% ash, and 1.6% volatile materials, with an enzyme activity of 2.8 AU/mg dry solids. Further characterization of the lyophilized test material revealed 0.15% lipids, 9.3% carbohydrates (as glucose equivalents), and 60% protein. Endotoxins were measured by kinetic chromogenic LAL assay to be 97,000 endotoxin units (EU)/mg. This corresponds to approximately 1.6% endotoxin by weight, as referenced against the USP Reference Standard Endotoxin for *Escherichia coli*. Chemical and microbiological analysis revealed: (a) no detectable heavy metals such as Pb, Cd, As, or Hg (<0.5 part per million); (b) absence of the *P. fluorescens* production microorganism; (c) total viable count less than 5×10^4 CFU/g; (d) no detectable *Salmonella* spp., coliforms, pathogenic, anaerobic, or microaerophilic microorganisms (FDA, 1998); (e) no detectable antibiotic activity (JECFA, 1992); and (f) absence of intact recombinant DNA/antibiotic resistance genes by Southern blot analysis (detection limit <1 part per billion).

The test material was prepared for gavage administration as an aqueous suspension of pH 7.0 ± 0.5 by

reconstitution of the lyophilized material in distilled water to 25% solids, with neutralization to approximately pH 7 with NaOH solution. This suspension was viscous and approximately the maximum suitable concentration that could be made and administered. The suspension was diluted with water as needed to prepare the target concentrations. The concentration of α-amylase was verified in all gavage and genotoxicity studies by enzyme activity measurement. Stability and homogeneity studies were conducted to validate the gavage procedures.

An analytical standard of BD5088 α-amylase enzyme, isolated and further purified from a typical production batch, was characterized using a variety of analytical and biochemical techniques. These techniques included, among others, enzyme activity assay, N- and C-terminal sequencing, peptide fingerprinting and mass coverage maps by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS), and molecular weight measurement (49.7 kDa) by liquid chromatography/mass spectrometry with electrospray ionization (LC-ESI MS). An isoelectric point of 4.5 was measured by capillary gel electrophoresis. The primary sequence of BD5088 α-amylase, as confirmed by the aforementioned techniques, consists of 435 amino acids (Table 1). The BD5088 enzyme included in the test materials used in the safety testing was identical to the analytical standard when compared by liquid chromatography, enzyme activity, SDS–PAGE isoelectric point (pI), molecular weight, and peptide coverage maps.

2.2. Experimental design

2.2.1. Pathogenicity evaluation of *P. fluorescens* biovar I

The pathogenic and toxigenic potential of orally administered *P. fluorescens* biovar I, strain MB101 was evaluated in Balb/c mice. MB101 is the parental strain of the *P. fluorescens* that is used to produce BD5088. The *P. fluorescens* strain with the BD5088 gene is DC88. Test material was administered by oral gavage in 0.2 ml of a uniform suspension of bacteria formulated on the basis of Colony Forming Units (6×10^8 or 1×10^8 CFU). Controls included sterile growth medium (uninoculated culture medium used to grow the test strain), filtered (0.22 μm) spent medium (culture medium after cells have been grown and removed), and uninoculated mice. Actively growing *P. fluorescens* cells were obtained from an overnight culture grown in a minimal salts medium with glucose as the sole carbon source. Mice were held for up to 21 days, with daily general observations of health. Subgroups of six bacteria-treated mice underwent necropsy on days 2, 4, and 7. The liver, spleen, mesenteric lymph node (MLN), large bowel, small bowel, and cecum were sampled for bacteria measurements. The subgroups of 6 control mice underwent necropsy on day 1. On the day of necropsy, all mice assigned for necropsy were evaluated by Detailed Clinical Observation (DCO), and body weights were determined. Also, all mice (bacteria-treated and controls) had DCO and body weight measurements on day 0.

For necropsy on day 2, 4, and 7, spleen, liver, MLN, large bowel, small bowel, and cecum were collected, weighed and homogenized for measurement on *Pseudomonas* Isolation Agar (PIA) for viable counts. Tenfold dilutions of the homogenized tissue were prepared in sterile saline, mixed thoroughly and 0.1 ml aliquots dispensed and spread evenly on PIA plates. Each dilution was plated in duplicate and the plates incubated at 30 ± 2 °C for 48 h. Colonies were counted on plates that resulted in a countable number of colonies and the number of CFU per gram of liver, spleen, MLN, large bowel, small bowel, and cecum was calculated.

2.2.2. Acute skin and eye irritation assessment

An aqueous suspension (22% TOS) adjusted to pH 7 was used to evaluate the effect of a near maximal concentration of test material. For the skin irritation study, 0.5 g of the test material suspension was applied to a test area (approximately 6.25 cm^2) on the back of each of three albino rabbits. A semi-occlusive dressing held the material in place. This was removed after 4 h and the area was washed with water and wiped dry. The skin was examined for erythema and swelling 0.5, 1, 24, 48, and 72 h after dressing removal. For the eye irritation study, 0.1 ml of the test material suspension was applied to one eye of each of three albino rabbits. Approximately 24 h later, the treated eyes were washed

Table 1
Primary amino-acid sequence of BD5088 α-amylase component enzyme

1	makyselekg	gvimqafywd	vpsggiwwdt	irqkipewyd	agisaiwipp
51	askgmggays	mgydpydffd	lgeydqkgtv	etrfgskqel	vnmintahay
101	gmkviadivi	nhraggdlew	npfvndytwt	dfskvasgky	tanyldfhpn
151	elhagdsgtf	ggypdichdk	swdqywlwas	qesyaaylrs	igidawrfdy
201	vkgyapwvvk	dwlnwwggwa	vgeywdtnvd	avlnwayssg	akvfdfalyy
251	kmdeafdnkn	ipalvsalqn	gqtvvsrdpf	kavtfvanhd	tdiiwnkypa
301	yafiltyegq	ptifyrdyee	wlnkdklknl	iwihenlagg	stdivyydnd
351	elifvrngyg	dkpglityin	lgsskagrwv	yvpkfagaci	heytgnlggw
401	vdkyvyssgw	vyleapaydp	angqygysvw	sycgvg	

Cys389 and Cys433 form a disulfide pair. The N-terminal methionine is cleaved after translation.

with saline to remove residual test material, if any. Eyes were examined at 1, 24, 48, and 72 h post-administration, with the use of sodium fluorescein dye at 24 h.

2.2.3. Allergenicity assessment

Amino-acid sequence homology: A sequence evaluation scheme based on those formulated by Gendel (1998a) and by the Joint FAO/WHO Expert Consultation (2001) was used to assess the similarity of the BD5088 α-amylase amino-acid sequence to known protein allergens. It has been recommended that an immunologically significant sequence identity requires a match of at least eight contiguous identical amino acids, or 35% identity over 80 amino-acid residues. GLP regulations were not applicable for this analysis.

Two protein allergen reference databases were generated. A non-redundant database (allnr v1.0.0; 1029 sequence entries) was generated by de-replicating entries retrieved from five published curated protein databases of known or putative allergen sequences (Table 2) and by searching the literature for published allergen sequences that have not been submitted to one of the public protein databanks (Swiss-Prot, PIR, GenPept). Sequences were de-replicated by Basic Local Alignment Search Tool (BLAST) analysis against the protein nr database at the National Center for Biotechnology Information (NCBI) to remove redundancies caused by accession of the same entry from different protein databanks.

A second comprehensive database (allotz v1.0.0; 4209 sequence entries) was created which contained all entries included in the first database prior to de-replication, as well as entries identified by keyword and BLAST searches. This includes (a) proteins identified by Entrez keyword searches of the protein database at NCBI (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db = Protein) using the keywords "allergen," "allergens," "isoallergen," and "isoallergens" and (b) proteins identified by BLAST searches of the non-redundant protein database at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) with significant similarity to proteins in the first database, where such proteins were from the same organism and were annotated with similar protein name or function. These databases include the known protein allergen TAKA-amylase, an α-amylase from *A. oryzae*.

In order to assess whether identity spanning eight contiguous amino-acid residues existed, all possible overlapping eight-letter words from the BD5088 component enzyme sequence were screened against each of the two allergen databases using the FINDPATTERNS program of the GCG Wisconsin Package version 10.2 (Genetics Computer Group, Madison, WI), allowing zero mismatched characters. To assess whether identity of at least 35% over any 80 amino-acid residues existed between the BD5088 component enzyme sequence and sequences contained in the two allergen databases, all possible overlapping 80 residue peptides from the BD5088 component enzyme sequence were screened against each of the two allergen databases using the FASTA program of the GCG Wisconsin Package version 10.2 with default parameters. Output alignments were screened for identity scores of 35% or greater with an overlap of the entire 80-residue length.

Pepsin digestibility: The digestibility of the BD5088 α-amylase analytical standard by pepsin was evaluated in vitro. Simulated gastric fluid (SGF) was prepared, containing porcine-derived pepsin and incubated with the test protein. The test incubations contained approximately 30,000 units peptidase activity and 250 μg test protein/ml incubation, as outlined by the United States Pharmacopoeia (USP, 2002). Reaction mixtures were prepared at pH 1.2 and 2.0 and incubated at 37 °C. The reaction mixtures were sampled periodically for up to 60 min, the aliquots neutralized, denatured, and the final digested samples analyzed by SDS–PAGE for characterization of intact protein and protein fragments. To determine whether precipitation of test protein occurred under the reaction conditions used, a 60-min sampling was neutralized with Na_2CO_3 and Laemmli buffer, warmed as above, and analyzed by SDS–PAGE for precipitated test protein.

2.2.4. Oral administration tests

A series of oral toxicity studies of increasing duration was performed in rats. The test animals received the enzyme preparation via gavage administration. On conclusion of these gavage studies, a 2-week dietary feeding study was conducted to clarify the significance of certain effects noted in the gavage studies. The following methodology was employed for all oral studies, except where noted otherwise.

Table 2
Published allergen databases included in both reference databases

Allergen database	Release/version	Internet location
IUIS (King et al., 1994)	4-Sep-2001	www.allergen.org
ILSI (Metcalfe et al., 1996)	N/A	N/A
BIFS (Gendel, 1998b)	v2	www.iit.edu/~sgendel/fa.htm
SwissProt Index of Allergens	24-Aug-2001	www.expasy.ch/cgi-bin/lists?allergen.txt
FARRP	v1.01	www.allergenonline.com

For all gavage and dietary feeding studies, rats were obtained from Charles River Breeding Laboratories (Raleigh, NC) and were acclimatized to the laboratory conditions for at least 1 week prior to administration of test material. Rats were 6–8 weeks of age at the start of all studies. Rats were fed certified lab chow ad libitum and were maintained on a 12 h photocycle with suitable housing conditions for temperature, humidity, and ventilation in an American Association for Accreditation of Laboratory Animal Care accredited facility.

Parameters evaluated included daily cage-side observations, detailed clinical observation, urinalysis, body weights, feed consumption, hematology, clinical chemistry, organ weights, and gross and histopathologic observations. Urine was collected prior to necropsy and was evaluated for volume, color, appearance, specific gravity and pH, bilirubin, glucose, proteins, ketones, blood and urobilinogen. The microsediment from pooled urine samples was evaluated microscopically.

At the scheduled necropsy, fasted animals underwent CO_2 narcosis. Blood samples were obtained from the orbital sinus and were analyzed for hematocrit, hemoglobin concentration, red blood cell count, total white blood cell count, platelet count, differential count, RBC indices (MCH, MCV, and MCHC), coagulation time, and prothrombin time. Serum samples were analyzed for the following enzyme activities: alkaline phosphatase, alanine aminotransferase, and aspartate aminotransferase. Concentrations were determined for albumin, cholesterol, creatinine, electrolytes (Na, K, PO_4, Cl, and Ca), glucose, total bilirubin, total protein, and urea nitrogen.

Animals were euthanized by decapitation and subjected to complete necropsy. The necropsy included examination of the external tissues and all orifices. The brain, pituitary, and adjacent cervical tissues were examined upon removal of the head. The eyes were examined in situ by application of a moistened microscope slide to each cornea. The nasal cavity was flushed via the nasopharyngeal duct and the lungs were distended to an approximately normal inspiratory volume with neutral, phosphate-buffered 10% formalin using a hand-held syringe and blunt needle. The skin was reflected from the carcass, the thoracic and abdominal cavities were opened and the viscera examined. All visceral tissues were dissected from the carcass, re-examined and selected tissues incised.

During necropsy, brain, liver, kidneys, heart, adrenals, uterus, ovaries, testes, epididymides, thymus, and spleen were trimmed and weighed immediately. The ratios of organ weight to terminal body weight were calculated. The following tissues were processed in formalin for histopathological examination: adrenals, aorta, bone (including joint), bone marrow, brain (cerebrum, brainstem, cerebellum), cecum, cervix, coagulating glands, colon, cranial nerve, duodenum, epididymis, esophagus, eyes, gross lesions, heart, jejunum, ileum, kidneys, lachrymal/harderian glands, larynx, liver, lungs, mammary glands (females), mediastinal lymph node, mediastinal tissues, mesenteric lymph node, mesenteric tissues, nasal tissues, oral tissues, ovaries, oviducts, pancreas, parathyroid glands, peripheral nerve, pituitary, prostate, rectum, salivary glands, seminal vesicles, skeletal muscle, skin and subcutis, spinal cord, spleen, stomach, testes, thymus, thyroid gland, trachea, urinary bladder, uterus, vagina.

Means and standard deviations were calculated for all continuous data. Body weights, feed consumption, organ weights, and appropriate hematologic data were evaluated by Bartlett's test ($\alpha = 0.01$) for equality of variances. Based on the outcome of Bartlett's test, exploratory data analysis was performed by a parametric or non-parametric analysis of variance (ANOVA). If significant at $\alpha = 0.05$, the ANOVA was followed respectively by Dunnett's test ($\alpha = 0.05$) or the Wilcoxon rank-sum test ($\alpha = 0.05$) with a Bonferroni correction for multiple comparisons with the control. Descriptive statistics only (means and standard deviations) were calculated for body weight gains, RBC indices, and differential WBC counts.

5-Day gavage study: BD5088 α-amylase preparation (0 and 790 mg TOS/kg/day) was administered by oral gavage (10 ml/kg) to 5 female Fischer rats for 5 consecutive days. Gavage administration was intended to provide a defined oral dose. The test material was reconstituted as an aqueous suspension from a lyophilized enzyme preparation. Amylase content was confirmed by enzyme assay. Parameters evaluated were as listed above.

14-Day gavage study: Groups of 10 rats per sex were given the test material at doses of 0, 71, 220, 710, or 2200 mg TOS/kg/day by gavage for 14 consecutive days. The volume administered was 10 ml/kg. α-Amylase in the dose suspension was shown by enzyme assay to be stable over a period of 17 days when refrigerated. Parameters were evaluated as described above, but with weekly clinical observations and additional ophthalmological observations. The eyes of all animals were examined by a veterinarian pre-exposure and prior to the scheduled necropsy using indirect ophthalmoscopy. Tissues were collected as described previously, with sample collection and necropsy after the 14-day administration period. Complete histopathological evaluations were carried out on all high-dose and control animals; based on results observed in these animals, liver and nasal tissues from animals dosed at the intermediate levels were also evaluated.

13-Week gavage study: Groups of 20 male and 20 female Fischer 344 rats were administered 0, 89, 270, or 890 mg BD5088 α-amylase/kg/day (as TOS) via oral gavage for 13 weeks to evaluate the potential for systemic toxicity. The material was administered as an

aqueous suspension in a volume of 5 ml/kg. The reduced dose volume for the 13-week study was intended to help reduce the possibility of direct contamination of the respiratory tract by the gavage procedure. An extra rat of each sex was assigned to each group to help ensure a full 20 rats would go through the entire study. Data, including pathology, were collected for all surviving rats.

Parameters examined were as described previously. In addition, cage-side clinical observations, weekly detailed clinical observations, ophthalmological exams, weekly body weights, weekly feed consumption, week 12 urinalysis, hematology, clinical chemistry, and organ weights were recorded. Blood samples for hematology and clinical chemistry were collected prior to necropsy. Gross necropsies were conducted on all rats. Histopathology was conducted on all rats from the control and maximum dose groups. In addition, lungs, nasal tissues, liver, kidneys, and relevant gross lesions were examined from the 89 and 270 mg/kg dose groups.

14-Day dietary feeding study: To investigate the significance of effects noted in the respiratory tracts of animals in the course of gavage studies, groups of 20 male and 20 female Fischer 344 rats were administered 0 or 310 mg BD5088 α-amylase/kg/day (as TOS) in the diet for 14 days. The test material was pre-mixed in a corn oil slurry, then mixed in the diet so that the final corn oil concentration was 1–2%. This procedure was used to minimize potential dusting that could occur when the lyophilized BD5088 preparation was mixed in the diet. (The commercial BD5088 preparation is not prepared in lyophilized form.) A suitable corn oil premix slurry could be prepared at this dose level without unduly modifying the oil content of the feed. Also, this dose level exhibited nasal tract effects in the 14-day gavage study.

Cage-side clinical observations, weekly body weights, and weekly feed consumption were evaluated. Blood samples for hematology were collected prior to necropsy. Gross necropsies and histopathology of the entire respiratory tract (nasal tissues to lung) were conducted on all rats. (Urine analysis and other organs and tissues were not evaluated, as this assessment focussed specifically on the respiratory tract. No adverse effects on other parameters had been observed in the gavage studies.) Liver samples were also examined histologically as some effects had been observed in the 5-day gavage study.

2.3. Genetic toxicology studies

Genetic toxicology evaluations of BD5088 α-amylase were designed to assess potential for causing mutation and chromosomal aberration in four assay systems that are commonly used for this purpose. Doses are reported as Total Solids derived from the enzyme preparation. The corresponding TOS levels may be determined based on a TOS content equivalent to 89% of the total solids. The enzyme was initially prepared as an aqueous suspension (in growth medium as appropriate) and BD5088 content was verified by enzyme assay.

(a) *Salmonella–Escherichia coli*/mammalian (Ames) microsome reverse mutation assay (treat and plate method). The tester strains used in the assay were *Salmonella typhimurium* TA98, TA100, TA1535, TA1537 (Ames et al., 1975; Maron and Ames, 1983), and *E. coli* WP2uvrA (Green and Muriel, 1976). The *Salmonella* and *E. coli* tester strains were histidine and tryptophan auxotrophs, respectively. Detection of mutations was based on reversion to histidine or tryptophan independence. The tester strain genotype for *rfa* wall mutation and pKM101 plasmid was confirmed on the day of testing. Assays were conducted with and without S9 mix, with appropriate controls. (Positive controls are identified in Table 7.) The S9 homogenate was prepared from the livers of male Sprague–Dawley rats injected with Aroclor 1254. (A similar S9 preparation was used in the mouse lymphoma and rat lymphocyte chromosome aberration tests.) Test material concentrations up to 5000 μg/ml were established based on a range-finding cytotoxicity study. Tester strains were exposed by the treat and plate method (Green and Muriel, 1976), which utilized a preincubation period of 60 ± 5 min at 37 °C, followed by a rinsing step to remove test article prior to plating. This procedure was used because histidine and tryptophan in the enzyme preparation might permit tester strain growth on the plates leading to overgrown background lawn and potential false positive responses. All concentrations and control articles were plated in triplicate and initial results were confirmed in an independent experiment. Concentrations of BD5088 in the test material stock solutions were confirmed by enzyme activity measurement. A positive test required a reproducible, concentration-dependent increase in mean revertants per plate relative to the appropriate control of at least twofold in TA100 and threefold in the other tester strains.

(b) Mouse lymphoma forward mutation assay: The ability of BD5088 α-amylase enzyme preparation to induce a mutation at the TK locus of mouse lymphoma cells was evaluated in three independent assays. These studies were conducted in accordance with OECD-guidelines (1997a), using the procedures described by Clive et al. (1987). L5178Y $TK^{+/-}$ cells were treated with 125–5000 μg/ml (as Total Solids per ml in Fischers medium) with and without S-9 metabolic activation system. Positive controls, 20-methylcholanthrene and methyl-methanesulfonate, were used for activation and non-activation assays, respectively. Following the addition of the test compounds, test tubes were incubated for approximately 4 h at 37 °C in a roller drum. At the end of the incubation period, the cells were pelleted, rinsed

with Fischers medium, and resuspended in 20 ml Fischers-based medium. The tubes were returned to the roller drum and maintained at 37 °C during a standard expression period of 2 days. Cultures were again counted and treatment levels with desired levels of toxicity were selected for cloning. Cultures with <10% Relative Suspension Growth on day 2 were not cloned. A total sample size of 3×10^6 cells from each culture was suspended in cloning medium with trifluorothymidine (TFT) and plated into three petri dishes, allowed to gel and returned to the incubator for approximately 12 days to allow for mutant colony formation. The cloning efficiency was determined by serially diluting the sample in cloning medium without TFT, plating the cells into three petri dishes and incubating for approximately 12 days. An image analyzer was used to count and size colonies. Criteria for a positive response include a statistically significant, reproducible dose-related increase in mutant frequency for a dose level range yielding at least 20% relative total growth and a mutant frequency in at least one dose level that exceeds the vehicle control by at least 100 per 10^6 cells (assuming control values in the range of 20–80 per 10^6 cells). Statistical assessment included analysis of variance (ANOVA) comparing all dose groups for each assay with equivalent activation ($\alpha = 0.05$).

(c) In vitro rat lymphocyte chromosomal aberration test utilized lymphocytes from Sprague–Dawley rats cultured in RPMI-1640 medium to evaluate clastogenic potential. Positive controls, mitomycin C and cyclophosphamide monohydrate, were used for non-activation and activation assays, respectively. An external metabolic activation system (S-9) was used to facilitate the identification of agents requiring biotransformation for clastogenic activity. This system was developed by Sinha et al. (1985, 1989) and met OECD 473 guideline requirements. Approximately 48 h after the initiation of whole blood cultures, cells in the absence and presence of S-9 activation were treated for 4 h with nine targeted concentrations of 0 (negative control) to 5000 μg BD5088 α-amylase preparation per ml of culture medium. The cultures were harvested 20 h later. As there was little or no toxicity based on mitotic indices, cultures treated with concentrations of 0, 1250, 2500, and 5000 μg/ml in the absence and presence of S-9 activation were selected for determining the incidence of chromosomal aberrations. Cells were evaluated for structural aberrations, including chromosome and chromatid breaks and exchanges, as well as for numerical aberrations (polyploidy). Gaps alone were not considered to represent structural aberrations. In a confirmatory assay, cultures were treated as above except that the cultures without S-9 were treated continuously for 24 h until the time of their harvest. In this second series, some toxicity occurred, so dose levels of 0, 125, 250, and 500 μg/ml, (non-activated) and 0, 1000, 2000, and 4000 μg/ml (S-9 activated) were tested for determination of chromosomal aberration. A positive test result required a reproducible, significant dose-related increase in the frequency of aberrant cells.

(d) The mouse micronucleus (MN) test is a short-term in vivo cytogenetic assay for detecting agents that induce chromosomal breakage and spindle malfunction (Mavournin et al., 1990; OECD, 1997b). The enzyme preparation was administered to male CD-1 mice by oral gavage on 2 consecutive days at dose levels of 0 (negative control), 500, 1000, and 2000 mg/kg body weight as enzyme preparation total solids in an aqueous suspension. Dosing solutions were administered as a split dose of two treatments (10 ml/kg) on the same day, separated by at least 3 h. The highest dose level of 2000 mg/kg was based upon the results of a range finding test and was the limit dose. Enzyme concentrations of the dosing solutions were verified by analytical methods. Groups of animals, 6 per dose, were sacrificed 24 h after treatment on the second day of dosing for the collection and evaluation of femoral bone marrow. Mice treated with 120 mg/kg cyclophosphamide monohydrate and sacrificed 24 h later served as positive controls. Two thousand polychromatic erythrocytes (PCE) were examined from each animal and the number of micronucleated PCE (MN-PCE) was recorded. As a measure of cell toxicity, the ratio of PCE to normochromatic erythrocytes (NCE) in the bone marrow was determined by examining 200 erythrocytes. A test was considered positive if a statistically significant increase in the MN-PCE frequency was observed at one or more dose levels accompanied by a dose–response.

3. Results

3.1. Pathogenicity evaluation of P. fluorescens biovar I

Elevated concentrations of *P. fluorescens* (6×10^8 or 1×10^8 CFU/mouse) were administered to female Balb/c mice in order to assess the potential health effects associated with oral exposure to the live organism. The ability of the test strain to infect mice was measured by the recovery of the dosed strain from selected organs and tissues. The results of these analyses for spleen, mesenteric lymph nodes, and liver are summarized in Fig. 1. Oral exposure of *P. fluorescens* resulted in detectable levels of pseudomonads in all mice examined, although significant heterogeneity was noted on day 2 CFU recovered on the selective medium (PIA) within each subgroup of mice. For example, the concentration of organisms recovered from liver ranged from non-detectable (<18 CFU/g of tissue) to 4.67×10^3 CFU/g of tissue. Not all tissues from infected mice contained detectable levels of organisms. On day 2, at the high

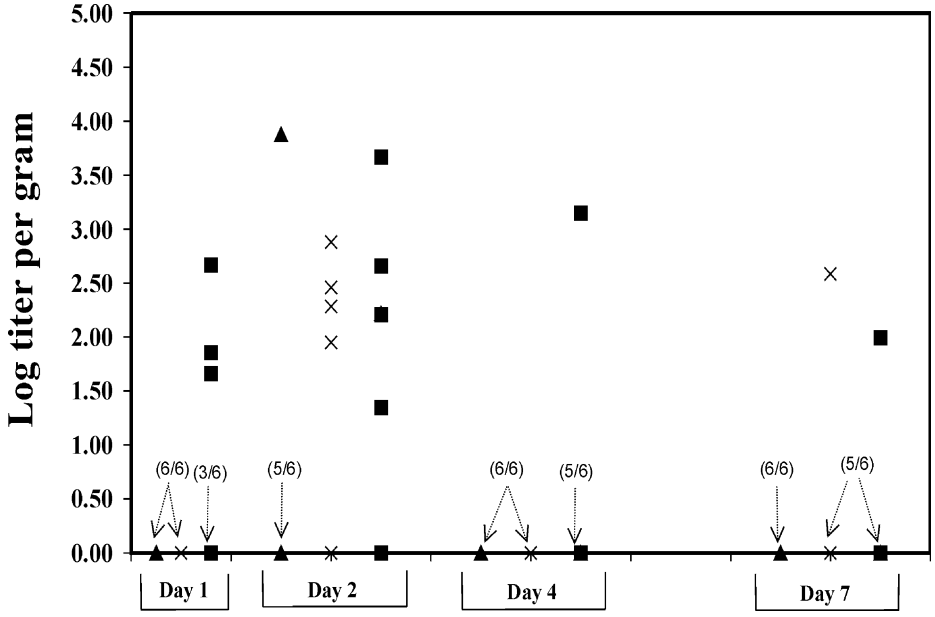

Fig. 1. Recovery of colony forming units (CFU) following oral treatment with *P. fluorescens* strain MB101 at a concentration of 6×10^8 CFU/mouse. Spleen (■), mesenteric lymph nodes (×), and liver (▲) were removed from the mice ($n = 6$) at 2, 4, and 7 days following treatment. Pseudomonads (not specific to *P. fluorescens*) were enumerated on PIA selective medium. Ratios indicated by arrows represent the number of mice where no CFU were detected in the designated tissue. Pseudomonads were enumerated from the spleen, mesenteric lymph nodes, and liver of the uninoculated control mice ($n = 6$) on day 1.

dose, pseudomonads were detected in tissues from 5 of 6 (liver), 4 of 6 (MLN), and 1 of 6 (spleen) mice. Five of six mice dosed at the lower concentration contained detectable levels of organisms in the spleen (data not shown). Elimination of the test strain from the liver, spleen, and MLN was noted 4 days post-treatment. As noted in Fig. 1, no CFU were recovered on day 4 from the spleen or MLN, and detectable levels of organisms were apparent in the liver of only one mouse. Similar results were noted for the liver, spleen, and MLN of the mice dosed at the lower (1×10^8 CFU/mouse) concentration (data not shown). Microorganisms capable of growth on Pseudomonas Isolation Agar (PIA) plates were also detected in the bowels (large and small) and ceca of mice from both dose groups. Elimination of the test strain from the bowels and cecum was difficult to discern since the normal microbial flora of the uninoculated control mice produced a high level of background CFU on PIA plates (Fig. 1).

In this study no mortality was observed over a 21 day period following oral exposure of Balb/c mice to *P. fluorescens* at either dose (6×10^8 or 1×10^8 CFU/mouse). Infection with *P. fluorescens* did not result in any clinical signs of morbidity such as ruffled fur or lethargy during the 21-day period. The animals appeared healthy and did not exhibit any weight loss, as the body weights of the infected animals were not significantly different from the uninoculated controls (data not shown). Similar results were noted for animals exposed to filter sterilized (0.22 μm) spent culture medium used to grow the inoculum for this study. No morbidity or mortality was noted for these animals and their weights were indistinguishable from the uninoculated controls.

3.2. Acute skin and eye irritation assessment

Animals did not exhibit any gross evidence of treatment-related toxicity (by observation) during either the skin or eye irritation study. For skin evaluation, very slight edema was noted in one rabbit at the 4 h observation period only (no erythema or corrosion). For eye irritation, there was no apparent pain. Slight conjunctival redness and chemosis were observed in all animals at 1-h post-administration. The treated cornea and iris appeared normal in each rabbit. All signs of irritation were resolved by 48 h, and there were no signs of corrosion at any time. The test material, in concentrated form relative to intended use, was slightly irritating to the skin and eye of rabbits.

3.3. Allergenicity assessment

Amino-acid sequence homology: According to suggested criteria an immunologically significant sequence identity between proteins requires a match of at least eight contiguous identical amino acids (Gendel, 1998a; Metcalfe et al., 1996) or an identity of 35% over 80 amino acids (FAO/WHO, 2001). The GCG FINDPATTERNS analysis revealed no identity of eight or

more contiguous amino-acid residues between the BD5088 sequence and any of the sequences in the two allergen databases. The GCG FASTA analysis revealed no identity of 35% or greater over 80 amino-acid residues between the BD5088 sequence and any of the sequences in the two allergen databases. Although this criterion was not met, there was some homology over the 80 amino-acid residue search window with TAKA-amylase. This may reflect the common enzymatic α-amylase functionality.

These assessments are used to evaluate whether there is a possibility that an introduced protein may share either a linear or conformational epitope with a known allergen. While such methods may provide an indication of an introduced protein's ability to cross-react with a known allergen and thereby elicit an allergic response, there are limits to the predictive capability of these methods to determine whether a protein is likely to function as a new allergen. On this basis, consideration of extremely low exposure potential is also a relevant consideration.

Pepsin digestibility: Incubation of BD5088 α-amylase analytical standard with pepsin at pH 1.2 resulted in rapid and complete digestion of the test protein within 0.5 min. The estimated digestion half-life for α-amylase under these conditions was less than 0.067 min. Incubation with pepsin at pH 2.0 also resulted in rapid digestion of the test protein. No intact protein remained at 0.5 min. Three minor protein fragments, each representing less than 3% of the starting material, were observed at 0.5 min. These protein fragments were not observed at 2 min or later time points. The estimated digestion half-life for α-amylase at pH 2.0 was less than 0.3 min. The results of these digestion experiments indicated that the BD5088 α-amylase protein is rapidly and completely digested by pepsin under standardized conditions that simulate mammalian gastric fluid. Digestibility in pepsin has been used as a screening factor in a decision tree for proteins to act as a food allergen (Metcalfe et al., 1996). Although *non*-digestibility has been considered a characteristic of food allergens, digestibility has not been proven to be predictive for identifying food allergens.

3.4. Oral administration tests

5-Day gavage study: Rats given the test material at a dose of 790 mg TOS/kg/day had a microscopic alteration in the intestinal tract, characterized by a loss of heterochromatin from epithelial nuclei, giving the cells a vesicular appearance. This occurred in most of the treated rats and most prominently in the rectum. A slight inflammatory reaction in the nasal tissues/turbinates was considered to be a result of the inadvertent reflux of test material into the nasal cavity during the gavage procedures. There were no other treatment-related changes based on observations, body weights, feed consumption, or clinical and anatomic pathology.

14-Day gavage study: Feed consumption was slightly decreased among male rats given the test material at doses of 710 and 2200 mg TOS/kg/day; males in the higher dose group, 2200 mg/kg/day, had statistically identified decreases in mean body weights, with a mean value 8.2% less than control at termination. Statistical increases in several relative organ weights (2200 mg/kg groups) were considered secondary to the decrease in body weight. There were several isolated observations of noisy respiration in rats receiving the test material at doses of 710 and 2200 mg/kg/day: this was considered to be a secondary effect of probable regurgitation. At the top dose of 2200 mg/kg/day, terminal serum cholesterol was increased and a very slight lipid vacuolation primarily of periportal hepatocytes was observed. The degree of vacuolation was not considered to be adverse. Local suppurative inflammation of the nasal mucosa was seen in the majority of males given 220 mg/kg and greater and of females given 71 mg/kg and greater. In rats given the top dose, nasal tissues of 3 males and 4 females had normal appearance. This focal response and lack of effect in some top dose rats was consistent with local irritation due to the gavage procedure, whereby small quantities of test material are likely to reflux into the nasal tract when the intubation needle is withdrawn. A small increase in mean spleen weight of female rats given 71, 710, and 2200 mg/kg/day was not considered to be treatment related due to the small magnitude (relative increase of 11% in top dose group), the range in comparison with historical values, and the lack of corresponding histopathology. There were no other treatment-related changes based on observations, organ weights, or pathology. Based on the decreased body weight in males given the test material at 2200 mg/kg/day, the NOAELs were 710 mg/kg/day in males and 2200 mg/kg/day in females.

13-Week gavage study: Three rats died during the study (2 males in the 89 mg/kg/day dose group and 1 female in the 890 mg/kg/day dose group), with lung inflammation and edema noted in the pathology examination. Based on these findings, the deaths were attributed to aspiration of the test material due to gavage error. The only treatment-related clinical observations were occasional noisy respiration, also considered to be gavage related. The occasional noisy respiration was transient, as it was not observed during the detailed clinical observations. There were no statistically identified treatment-related effects on body weights (see Fig. 2) or urinalysis parameters (data not shown).

Clinical chemistry and hematology results are summarized in Table 3. There was a statistically identified 14% increase in alkaline phosphatase (ALP) among males dosed at 890 mg/kg/day (mean ALP value of 117 ± 13 U/l in high-dose males compared with

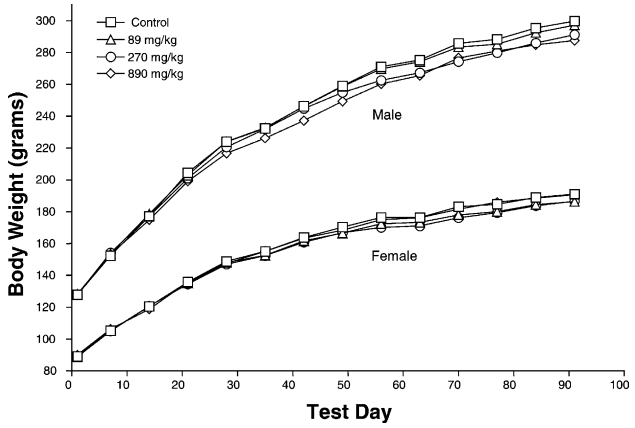

Fig. 2. Mean body weights of rats administered BD5088 α-amylase for 13 weeks.

103 ± 11 U/l in male controls). However, ALP values remained within the range of historical control values for the testing laboratory of 95–131 U/l. Moreover, no other liver effects were found in these animals. For these reasons, the increase in ALP was not interpreted as a treatment-related effect. Statistical decreases (ALT and AST) in 89 mg/kg treated females were not consistent with a dose–response relationship and were not considered treatment related. In hematology, there were no statistically identifiable changes based on overall treatment group comparisons. However, an increase in neutrophil count was observed among male rats dosed at 890 mg/kg/day; this increase was associated with exudative mucopurulent nasal inflammation, as discussed further below.

Group mean organ weights are summarized in Fig. 3. The only statistically identified change in organ weights that may have been treatment related was an increase in mean absolute and relative liver weight among females dosed at 890 mg/kg/day. (Mean absolute and relative liver weights in dosed females: 5.168 g and 2.909 g/100 g; mean absolute and relative liver weights in controls: 4.740 g and 2.662 g/100 g.) Although liver weights in these animals were above those of historical controls (absolute liver weights, 3.870–4.587 g; relative liver weights, 2.410–2.750 g/100 g), the difference was not considered to represent a significant biological effect as there were no corresponding histopathological liver effects.

Some rats from both sexes and all treatment groups had very slight to slight chronic active inflammation of the nasal mucosa (respiratory and/or olfactory). Some of the male rats given 270 or 890 mg/kg/day and 1 female given 270 mg/kg/day had mucopurulent exudates in the nasal cavities. Additionally, some of the rats of both sexes and from all treatment groups had very slight to slight, focal to multifocal, subacute to chronic inflammation of the lung. The incidence of animals with respiratory tract lesions is summarized in Table 4. Representative photomicrographs of the nose and lung lesions are provided in Fig. 4. These nasal and lung effects were interpreted to be related to reflux of the test material into the nasal cavity and/or aspiration into the lung. Because the enzyme preparation was made with a

Table 3
Selected hematology and clinical chemistry parameters in rats treated with BD5088 α-amylase for 13 weeks

Parameter	Males				Females			
	0 mg/kg	89 mg/kg	270 mg/kg	890 mg/kg	0 mg/kg	89 mg/kg	270 mg/kg	890 mg/kg
UN (mg/dl)	14 ± 1	14 ± 2	14 ± 2	14 ± 2	13 ± 2	13 ± 1	14 ± 1	13 ± 2
ALT (U/l)	67 ± 21	61 ± 10	65 ± 42	60 ± 21	50 ± 14	39 ± 7*	45 ± 13	46 ± 12
ALP (U/l)	103 ± 11	106 ± 14	108 ± 12	117 ± 13*	82 ± 10	84 ± 10	87 ± 9	89 ± 12
AST (U/l)	102 ± 16	97 ± 12	104 ± 47	101 ± 20	98 ± 27	79 ± 10*	89 ± 15	90 ± 26
GLUC (mg/dl)	133 ± 23	128 ± 16	124 ± 14	124 ± 17	126 ± 33	122 ± 22	120 ± 19	116 ± 16
TP (g/dl)	6.7 ± 0.2	6.8 ± 0.3	6.7 ± 0.2	6.7 ± 0.2	6.4 ± 0.3	6.5 ± 0.2	6.4 ± 0.2	6.4 ± 0.2
ALB (g/dl)	3.4 ± 0.1	3.4 ± 0.1	3.4 ± 0.1	3.4 ± 0.1	3.4 ± 0.1	3.4 ± 0.1	3.3 ± 0.1	3.3 ± 0.1
CHOL (mg/dl)	57 ± 8	59 ± 14	54 ± 8	54 ± 8	86 ± 10	83 ± 11	82 ± 9	82 ± 8
WBC (10^3/μl)	10.1 ± 1.6	10.0 ± 1.6	10.7 ± 1.3	10.8 ± 1.9	8.8 ± 2.0	9.4 ± 1.2	8.6 ± 1.6	9.5 ± 1.9
RBC (10^6/μl)	8.6 ± 0.3	8.4 ± 0.3	8.6 ± 0.3	8.6 ± 0.2	8.0 ± 0.3	7.9 ± 0.3	8.0 ± 0.2	7.9 ± 0.2
HGB (g/dl)	15.4 ± 0.3	15.3 ± 0.5	15.2 ± 0.5	15.1 ± 0.5	15.4 ± 0.6	15.3 ± 0.4	15.3 ± 0.4	15.2 ± 0.4
PLT (10^3/μl)	621 ± 55	649 ± 58	636 ± 51	640 ± 60	670 ± 40	653 ± 43	661 ± 52	644 ± 48
NEUT (%)	16.1 ± 2.1	16.9 ± 3.0	18.4 ± 4.4	20.4 ± 5.6	15.7 ± 3.2	16.8 ± 3.1	15.9 ± 3.2	16.6 ± 3.5
LYMP (%)	77.5 ± 2.5	76.6 ± 3.6	75.0 ± 5.1	73.4 ± 6.1	77.5 ± 4.2	75.6 ± 4.0	77.5 ± 4.2	76.0 ± 3.8
MON (%)	2.8 ± 0.5	2.7 ± 0.6	2.8 ± 0.6	2.6 ± 0.5	2.8 ± 0.8	2.9 ± 0.7	2.5 ± 0.6	2.8 ± 0.6
EOS (%)	0.9 ± 0.2	0.9 ± 0.2	0.9 ± 0.2	0.9 ± 0.3	1.0 ± 0.3	1.1 ± 0.3	1.0 ± 0.3	1.0 ± 0.2
BASO (%)	0.5 ± 0.2	0.6 ± 0.2	0.7 ± 0.2	0.6 ± 0.2	0.6 ± 0.3	0.6 ± 0.3	0.6 ± 0.3	0.7 ± 0.2
LUC (%)	2.0 ± 0.4	2.3 ± 0.6	2.3 ± 0.6	2.2 ± 0.6	2.5 ± 0.6	3.0 ± 0.8	2.5 ± 0.8	3.0 ± 0.6

Values are the mean of 20–21 rats ± 1SD. UN, urea nitrogen; ALT, alanine aminotransferase; ALP, alkaline phosphatase; GLUC, glucose; TP, total protein; ALB, albumin; CHOL, cholesterol; WBC, white blood count (10^3/μl); RBC, red blood count (10^6/μl); HGB, hemoglobin (g/dl); PLT, platelets (10^3/μl); NEUT, neutrophils (%); LYMP, lymphocytes (%); MONO, monocytes (%); EOS, eosinophils (%); BASO, basophils (%); and LUC, large unstained cells.

* Statistically identified difference from control $p < 0.05$, differential count values were not statistically compared.

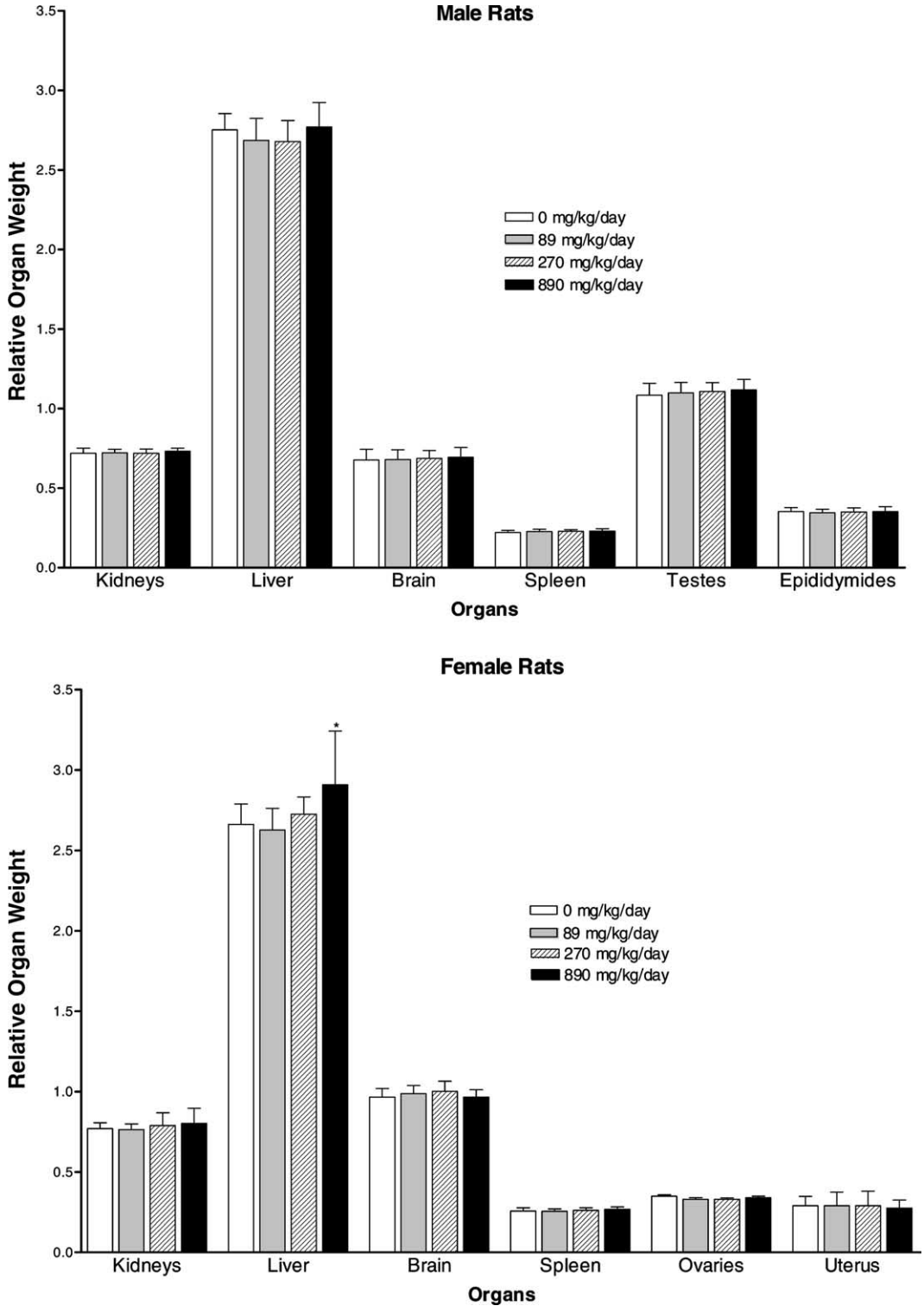

Fig. 3. Selected relative organ weights (grams per 100 grams of body weight) in rats administered BD 5088 α-amylase preparation for 13 weeks by gavage. Relative ovary weights are in g/1000 g. Values are means ± 1SD. *The mean liver weight of females (890 mg TOS/kg/day) was the only statistically identified difference from the respective control (Wilcoxon rank sum test, $\alpha = 0.05$).

Gram negative production organism, it contained lipopolysaccharide (endotoxin); inhalation or instillation of endotoxins can cause inflammation of the respiratory tract (Gordon and Harkema, 1994).

There were three measurements of weekly feed consumption in 890 mg/kg/day treated male rats that were statistically decreased; these were considered to be related to the nasal inflammation. Exudative nasal in-

Table 4
Overall incidence of respiratory tract lesions in rats administered BD5088 α-amylase preparation for 13 weeks by gavage

	Males				Females			
	0 mg/kg	89 mg/kg	270 mg/kg	890 mg/kg	0 mg/kg	89 mg/kg	270 mg/kg	890 mg/kg
Lung	0	9	16	9	0	1	10	7
Nasal Cavity	3	11	15	9	5	16	15	8

Histopathological changes of the lung included very slight, slight or severe, acute or subacute to chronic and focal or multifocal inflammatory lesions of the bronchiolo-alveolar regions of the lung. Changes of the nasal cavity included very slight or slight, chronic active, focal or multifocal and mucopurulent exudative inflammatory changes of the respiratory and/or olfactory mucosa. Number of rats evaluated = 21/sex/dose group.

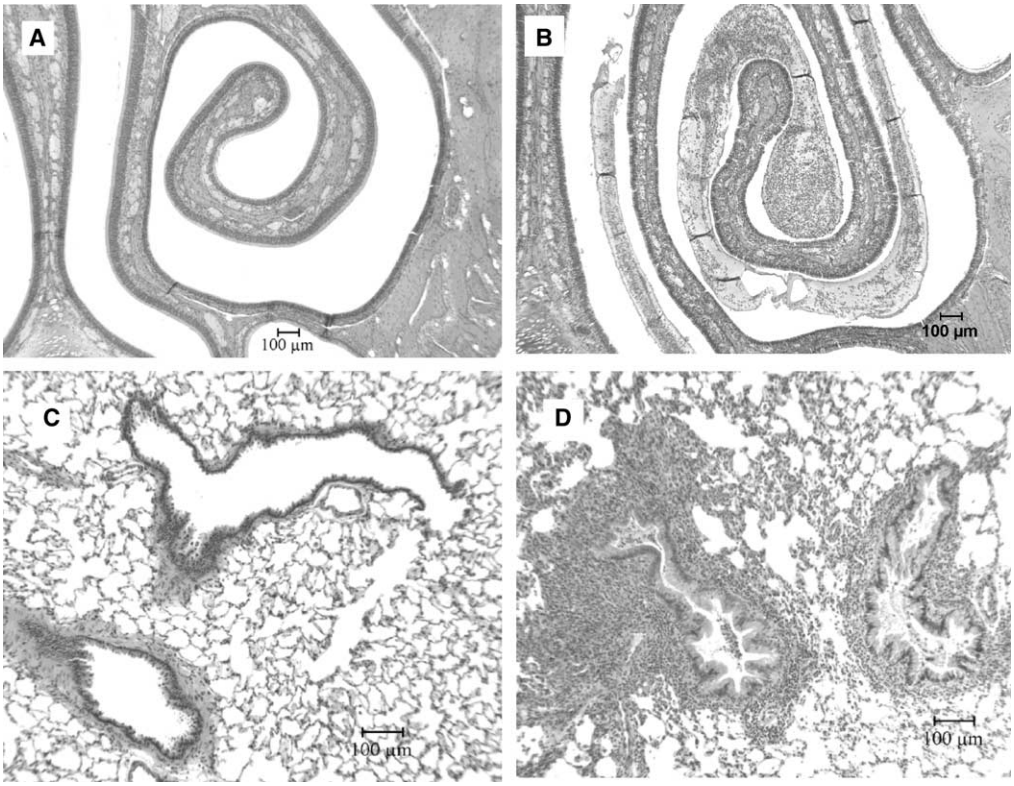

Fig. 4. H&E stained sections of nose and lung from rats given 0 (A or C) or 890 or 89 mg TOS/kg/day BD5088 α-amylase for 90 days (B and D, respectively). (B) Exhibits nasal lumen filled with mucopurulent exudate. (D) Exhibits peribronchiolar infiltrate of lymphocytes, macrophages, and neutrophils. The inflammatory cells are also seen within the lumen of the bronchioles. Note the increased height of the lining bronchiolar epithelium indicative of regenerative hyperplasia.

flammation was associated with decreased feed consumption and increased circulating neutrophils in individual males given 890 mg/kg/day (Table 5). Individual comparisons were considered more relevant than statistical comparisons for both neutrophil % and feed consumption. Feed consumption was not consistently affected in the treatment groups. Absolute blood neutrophil concentrations were affected similarly (data not shown) and in the proportion of neutrophil to total white cell count (Table 3). Female rats treated with 890 mg/kg/day did not exhibit nasal exudative inflammation, so individual comparison with neutrophil count was not made.

Myeloid cell hyperplasia was observed in three males (890 mg/kg/day); this was considered secondary to the respiratory inflammation. Increased observations of white blood cells in urine of males given 270 or 890 mg/kg/day were noted (not a statistical comparison). There was no corresponding urinary or renal histopathology. If exposure related, the small change in urine WBC was considered to be of minimal biological significance in the context of this study. Treatment-related pathologic effects were limited to the respiratory tract. Body weights were not affected to a degree that was statistically identifiable, although feed consumption was affected at times in individual rats. Although some rats from all treatment groups had a degree of local respiratory tract inflammation, there were no adverse direct systemic effects considered to be related to the oral route of administration.

Table 5
Feed consumption and circulating neutrophils in male rats given 890 mg/kg/day by oral gavage

Nasal tissue: within normal limits			Nasal tissue: mucopurulent exudative inflammation		
Animal ID #	Feed consumption week 13 (g)	Neutrophil%	Animal ID #	Feed consumption week 13 (g)	Neutrophil%
5836	15.5	17.5	5837	10.1	25.2
5839	11.6	13.0	5838	9.7	27.8
5841	14.5	16.2	5844	11.8	32.1
5843	14.2	14.8	5846	12.6	27.5
5845	14.6	19.0	5850	11.8	28.5
5849	14.1	19.6	5851	13.0	21.6
5852	14.4	16.3	5853	10.3	28.8
5854	14.6	19.2			
5855	12.9	13.7			
5856	16.1	17.2			
Mean ± SD	14.3 ± 1.3	16.7 ± 2.3		11.3 ± 1.3	27.4 ± 3.3

Rats with nasal tissue that is within normal limits versus rats with exudative nasal inflammation. Males given 0 mg/kg/day consumed 15.1 g ± 1.1 of feed during week 13 and had 16.1 ± 2.1 neutrophil% at necropsy. Not all rats given 890 mg/kg/day were used in this assessment. Only those rats which were identified with normal nasal tissue, or those found with mucopurulent exudative inflammation were selected for this evaluation.

Based on the results of this study, the NOAEL for BD5088 α-amylase enzyme preparation (TOS basis) in Fischer 344 rats of either sex was considered to be the top dose administered, 890 mg/kg/day.

14-Day oral feeding study: Administration of BD5088 α-amylase enzyme preparation in the diet at a level of 310 mg/kg/day (TOS basis) did not result in any treatment-related effects on body weights, food consumption, or cage-side clinical observation. As evidenced by the data provided in Table 6, no treatment-related effects on hematology, including neutrophils, were observed. Further, gross and histopathologic examination of the respiratory tract and liver did not reveal any macroscopic or microscopic treatment-related changes in these tissues.

In the 14-day and 90-day gavage studies discussed above, localized inflammatory lesions were observed in the nasal mucosa and/or lungs of treated animals. These lesions were interpreted to be due to reflux and/or aspiration of the test material into the nasal cavity or lungs during the course of repeated oral gavage dosing. Hence, these results were interpreted to be not relevant to identifying the effects of BD5088 α-amylase when it is ingested as a component of the diet. In this feeding study, such inflammatory changes of the lungs and nasal cavity were not observed, thus confirming that the inflammatory effects observed in the previous studies were due to the gavage route of administration and were not associated with ingestion of BD5088 α-amylase enzyme preparation residues in food.

3.5. Genetic toxicology studies

Genetic toxicology evaluations of BD5088 α-amylase were designed to assess the potential for enzyme preparation constituents to induce point mutation and/or chromosomal aberration. A total of four assay systems were investigated to provide information on a wide range of potential genotoxic mechanisms.

Table 6
Selected hematology parameters in rats treated with BD5088 amylase in the diet for 2 weeks

Parameter	Males		Females	
	0 mg/kg	310 mg/kg	0 mg/kg	310 mg/kg
WBC (10^3/μl)	8.82 ± 1.44	9.30 ± 1.2	7.97 ± 1.26	8.50 ± 1.65
RBC (10^6/μl)	8.45 ± 0.31	8.36 ± 0.31	8.28 ± 0.28	8.28 ± 0.24
HGB (g/dl)	15.9 ± 0.6	15.7 ± 0.6	16.1 ± 0.4	16.0 ± 0.5
PLT (10^3/μl)	774 ± 90	815 ± 69	773 ± 45	773 ± 53
NEUT (%)	18.7 ± 2.1	17.7 ± 2.4	16.1 ± 3.4	15.2 ± 3.2
LYMP (%)	76.6 ± 2.5	77.9 ± 2.7	79.2 ± 3.5	80.0 ± 3.5
MON (%)	2.1 ± 0.5	1.9 ± 0.3	1.6 ± 0.6	1.7 ± 0.5
EOS (%)	0.8 ± 0.3	0.7 ± 0.3	0.9 ± 0.3	0.9 ± 0.2
BASO (%)	0.6 ± 0.2	0.6 ± 0.2	0.9 ± 0.2	0.8 ± 0.2
LUC (%)	1.3 ± 0.3	1.2 ± 0.3	1.3 ± 0.3	1.4 ± 0.3

Values are the mean of 20–21 rats ± 1SD. WBC, white blood count; RBC, red blood count; HGB, hemoglobin; PLT, platelets; NEUT, neutrophils; LYMP, lymphocytes; MONO, monocytes; EOS, eosinophils; BASO, basophils; and LUC, large unstained cells. There were no statistically identified differences from control values $p < 0.05$, differential count values were not statistically compared.

(a) *Salmonella–E. coli*/mammalian (Ames) microsome reverse mutation assay results are summarized in Table 7. BD5088 α-amylase enzyme preparation did not cause a positive increase in the reversion rate in any of the tester strains, with or without S-9 microsomal enzymes from Aroclor-1254 induced Sprague–Dawley rat liver. Acceptable results were obtained with positive and negative (vehicle) controls. Thus, it may be concluded that the enzyme preparation is non-mutagenic in this test system.

(b) Mouse lymphoma forward mutation assay: The protocol for this study called for two assays to be carried out on the BD5088 α-amylase enzyme preparation, an initial and a confirmatory assay. However, in order to reach the desired level of toxicity (relative total growth of 10–20%), the confirmatory assay was repeated. Thus, a total of three independent assays were conducted both in the absence and in the presence of metabolic activation. Tables 8–10 provide results for the first assay and the two confirmatory assays. In some of these experiments, statistically significant increases in mutant frequencies were observed, primarily at excessively toxic dose levels. However, in none of the experiments did such increases satisfy the criteria for a positive mutagenic response in this test system, because in no case was the mutant frequency average at any dose level greater than the concurrent vehicle control by a margin of at least 100 per 10^6 cells. Clive et al. (1995) had noted this criteria, including an assumption that concurrent controls are in the range of $20–80 \times 10^{-6}$, and importantly, that the experimenter should be aware of biologically irrelevant effects in severely stressed cells. Under the experimental conditions used, BD5088 α-amylase was considered to be non-mutagenic in the absence or presence of S-9 activation (from Aroclor-1254 induced Sprague–Dawley rat liver) in the mouse lymphoma forward mutation assay.

Table 7
Reverse mutation test in strains of *Salmonella typhimurium* and *E. coli*

Strain	Mean revertant colonies per plate									
	TA98		TA100		TA1535		TA1537		WP2 uvrA	
	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9	−S9	+S9
0 μg	23	30	94	104	9	14	9	10	136	172
10 μg	27	28	104	99	9	12	6	8	152	163
33 μg	25	27	106	105	12	17	6	8	143	163
100 μg	25	29	90	103	10	14	9	8	137	164
333 μg	24	29	101	115	13	14	8	11	149	168
1000 μg	23	27	106	119	14	14	7	10	151	193
5000 μg	11	15	85	85	10	9	5	9	109	148
Positive control	1822	1233	1697	1774	2237	111	5078	165	3013	674

Dose is in μg per ml of treat and plate reaction mixture. Results are the average of two independent assays, each in triplicate. S9 positive controls were treated with benzo(a)pyrene (TA98), 2-aminoanthracene (TA100, TA1535, TA1537, and WP2uvrA). Positive controls without S9 were 2-nitrofluorene (TA98), *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (TA100, TA1535), ICR-191 (TA-1537), and 4-nitroquinoline-*N*-oxide (WP2uvrA).

Table 8
Summary of mouse lymphoma BD5088 α-amylase first assay

Concentration (μg/ml)	Non-activated		S-9 Activated	
	% Survival (relative total growth)	Mutant frequency ($\times 10^{-6}$)	% Survival (relative total growth)	Mutant frequency ($\times 10^{-6}$)
0	105	77	89	97
0	95	94	Lost-contaminated	Lost-contaminated
500	96	86	Lost-contaminated	Lost-contaminated
500	104	77	115	82
1000	70	85	124	81
1000	75	82	91	85
2000	57	97	101	85
2000	59	90	87	97
3000	9	177[a]	21	123
3000	13	164[a]	11	173
3500	7	183	Excessive toxic	Not counted
3500	13	123	Excessive toxic	Not counted
Positive control	30	957[a]	15	616[a]
Positive control	30	732[a]	4	Not counted

In both series, at concentrations of 4000–5000 μg/ml, cells were not plated due to excessive toxicity based on day 2 Relative Suspension Growth.
[a] Significantly higher than concurrent control at $\alpha = 0.02$.

Table 9
Mouse lymphoma BD5088 α-amylase first confirmatory assay

Concentration (μg/ml)	Non-activated		S-9 Activated	
	% Survival (relative total growth)	Mutant frequency ($\times 10^{-6}$)	% Survival (relative total growth)	Mutant frequency ($\times 10^{-6}$)
0	101	72	111	107
0	99	80	90	112
125	83	94	106	103
125	93	87	90	87
250	71	87	119	83
250	84	78	111	92
500	(Not plated)	(Not plated)	107	109
500	140	50	101	97
1000	61	85	104	88
1000	68	90	84	86
2000	45	105[a]	65	105
2000	38	105[a]	84	90
Positive control	24	562[a]	14	821[a]
Positive control	10	912[a]	7	[b]

In the non-activation assay, one of the 500 μg/ml series was inadvertently not plated. In both series, at concentrations of 3000–5000μg/ml, cells were not plated due to excessive toxicity based on day 2 RSG.

[a] Significantly higher than concurrent control at $\alpha = 0.02$.

[b] Plates not counted due to excessive toxicity.

Table 10
Mouse lymphoma BD5088 α-amylase second confirmatory assay

Concentration (μg/ml)	Non-activated		S-9 Activated	
	% Survival (relative total growth)	Mutant frequency ($\times 10^{-6}$)	% Survival (relative total growth)	Mutant frequency ($\times 10^{-6}$)
0	102	61	95	97
0	94	49	103	84
125	98	64	89	110
125	95	75	102	88
250	103	43	113	84
250	87	55	106	79
500	89	59	75	111
500	78	65	63	105
1000	68	65	70	102
1000	73	75	82	108
2000	48	80	37	151[a]
2000	48	67	51	122[a]
2300	39	97[a]	14	179[a]
2300	28	93[a]	5	Not counted
2600	17	115[a]	Excessive toxic	Not counted
2600	21	115[a]	Excessive toxic	Not counted
Positive control	40	476[a]	77	332[a]
Positive control	23	684[a]	35	581[a]

In both series, at concentrations of 3000–5000μg/ml, cells were not plated due to excessive toxicity based on day 2 RSG.

[a] Significantly higher than concurrent control at $\alpha = 0.02$.

(c) In vitro rat lymphocyte chromosome aberration test: There were no statistically significant increases in the frequencies of cells with structural aberrations, or in the frequencies of polyploid cells in either assay, either in the absence or in the presence of metabolic activation. Results of 4 and 24 h treatment duration studies are summarized in Table 11. Cultures treated with the positive control chemicals (i.e., mitomycin C or cyclophosphamide) had significantly higher incidences of abnormal cells in all assays. Based on these results, BD5088 α-amylase was considered to be not genotoxic in this in vitro chromosomal aberration assay.

(d) Mouse micronucleus test: No treatment-related clinical signs were noted throughout the 48 h observation period. There were no statistically significant increases in the frequencies of MN-PCE for groups treated with the test material as compared to the negative controls (Table 12). The positive control animals showed a significant increase in the frequency of MN-PCE as compared to the negative control animals. There

Table 11
Rat lymphocyte chromosome aberration test, with results reflecting the scoring of 200 cells

Treatment duration	Without S-9 activation			With S-9 activation		
	Dose (μg/ml)	% Polyploid	% Cells with aberrations	Dose (μg/ml)	% Polyploid	% Cells with aberrations
4 h	0	0.0	0.5	0	0.0	1.5
	1250	0.0	1.0	1250	0.5	0.5
	2500	0.0	0.5	2500	0.0	1.5
	5000	0.0	0.5	5000	0.0	0.0
	MMC	0.0	31*	CP	0.0	15*
24 h	0	0.0	0.5	0	0.0	0.0
	125	0.0	1.0	1000	0.5	0.5
	250	0.5	1.5	2000	0.0	0.5
	500	0.0	1	4000	0.0	1.0
	MMC	0.0	23*	CP	0.0	15*

Positive controls were mitomycin C (MMC, 0.5 μg/ml) and cyclophosphamide monohydrate (CP, 4 μg/ml).
* Statistically identified, $p < 0.05$.

Table 12
Frequencies of micronucleated polychromatic erythrocytes (% MN-PCE) and polychromatic erythrocytes (% PCE)

Dose (mg/kg/day)	% MN-PCE Mean ± SD	% PCE Mean ± SD
0	1.7 ± 0.9	68.4 ± 2.8
500	2.1 ± 1.0	66.3 ± 11.3
1000	1.6 ± 0.7	59.8 ± 4.5
2000	1.7 ± 1.2	70.3 ± 5.3
120 CP	63.1 ± 13.7*	49.9 ± 11.8*

Values represent 2000 cells counted per animal (for % MN-PCE), with 200 erythrocytes evaluated for % PCE. Cyclophosphamide monohydrate (CP) was significantly different (*) from the negative control at $\alpha = 0.05$.

were no statistically significant differences in the percent PCE for groups treated with the test material while the mean percent PCE value of the positive control was significantly lower than the negative controls. Under the experimental conditions used, BD5088 α-amylase enzyme preparation was considered to be negative in the mouse bone marrow micronucleus test.

4. Discussion and conclusions

Bacterial clearance requires an intact and functional immune system that incorporates a cascade of immune responses. Bacterial clearance also provides an indication of the interaction between the potential pathogenicity of the invading microorganism and the total host immune capability. In the present study, oral administration of high doses of *P. fluorescens* MB101 parental strain resulted in translocation of the test strain to the MLN, spleen and liver of adult male Balb/c mice. The test strain did not appear to be virulent, and it was concluded that the organisms were eliminated from these tissues within four days of exposure. Similar results were reported by George et al. (2000), where rapid clearance of *P. fluorescens* from the MLN, spleen, and liver in male CD-1 mice treated orally with high levels ($\sim 10^8$ CFU/mouse) of this microbial agent was observed. They also noted some mortality at extremely high levels (approximately 10^9 bacteria/mouse) following intra nasal administration.

Inflammation in the respiratory tract in the BD5088 gavage studies was considered to be a local effect based on its focal nature, lack of dose–response and the occurrence of some rats with normal respiratory tract at all dose levels. There was not a large component of lymphocytes nor macrophages, nor any eosinophils in the inflammatory infiltrate to the nasal mucosa. Therefore, the inflammatory response was not characteristic of an allergic response.

Results of the 14-day feeding study indicated that oral administration in the diet was not associated with respiratory tract inflammation. In rats receiving the enzyme preparation by gavage, inflammation was consistent with the introduction of LPS (lipopolysaccharide or endotoxin) from *P. fluorescens* by direct contact with the respiratory tract. A second expert pathologist examined respiratory tract slides from the 13-week gavage and 2-week diet studies and confirmed that the inflammation was similar to that caused by LPS administered to rodents (Harkema, 2002).

Lipopolysaccharides are cell wall components of Gram-negative bacteria, to which both *P. fluorescens* and *E. coli* belong. LPS is ubiquitous at small levels throughout the environment, including house dust (Peterson et al., 1964). Inhalation of endotoxins can elicit acute neutrophilic inflammation of the airways (Gordon and Harkema, 1994). Laboratory rodents exposed to endotoxin by inhalation or intranasal instillation rapidly develop inflammation in nasal and pulmonary airways with intraluminal mucus secretion. Another rodent study demonstrated that the intratrac-

heal instillation of endotoxin (500 μg; *Pseudomonas aeruginosa*, serotype 10) can induce acute, transient, neutrophilic inflammation that precedes the appearance of mucous secretory cells in the pulmonary airways of F344 rats (Steiger et al., 1995).

In humans, aerosol inhalation of endotoxin from *E. coli* has been experimentally demonstrated to cause respiratory inflammation (Sandstrom et al., 1992). Because Gram-negative organisms, particularly *E. coli*, are common in the human gastrointestinal tract and humans are routinely exposed to small amounts of endotoxin, the possibility of small residual quantities of LPS in corn wet milling products presents a negligible exposure risk. Berczei et al. (1968) indicated that oral administration of *E. coli*-derived LPS had no effect, even on sensitive species, when given in doses from 500 to 3000 times the minimum parenterally lethal dosage.

The fermentation and recovery process as described in Section 2 will be used for commercial production, except that the recovery process has been further refined since preparation of the test materials. Improvements in filtration have reduced LPS levels at least 100-fold, to less than 50 ppm in the final enzyme preparation (relative to an *E. coli* LPS standard). Therefore, from the perspective of worker safety, commercial BD5088 material will have markedly less potential for respiratory tract irritation by direct contact than the cruder test materials utilized for the toxicity studies.

The safety of BD5088 α-amylase enzyme preparation in starch hydrolysis is further supported by the fact that enzyme preparation residues are expected to be present in finished starch hydrolysis products at extremely low levels, if at all, due to the repeated purification steps to which such products are subjected. The processing of corn starch, for example, to produce ingredients such as high-fructose corn syrup (HFCS), glucose syrup, and crystalline glucose, includes a series of purification steps intended to remove both unwanted organic and inorganic components. These steps include both cationic and anionic exchange as well as activated carbon and diatomaceous earth treatments.

Analysis of glucose syrups, produced with the aid of BD5088 α-amylase and processed under conditions and with equipment commonly utilized by the sweetener industry, yielded no detectable enzyme at a limit of detection (LOD) of 2.4 ppb, as measured by sandwich ELISA (enzyme-linked immunosorbent assay). On this basis, the maximum level of enzyme preparation residues in the syrup may be roughly estimated assuming (1) residues of the inactivated enzyme are present at one-half the LOD, or 1.2 ppb, consistent with common practice when an analyte is uniformly non-detected; (2) other components of the enzyme preparation TOS are removed during purification to the same extent as the enzyme; and (3) the enzyme comprises no more than 10% of the TOS. On this basis, enzyme preparation TOS residues in the finished sweetener will be no more than: 1.2 ppb enzyme × (100 parts TOS/10 parts enzyme), or 12 ppb.

Total consumption of enzyme preparation residues may then be estimated based on the additional assumption that all corn sweeteners in the United States are produced using the BD5088 enzyme preparation. As of 2001, total corn sweeteners (including HFCS, glucose, and dextrose) were consumed at an annual rate of 84 pounds per person, which corresponds to 104 g/person/day, according to Corn Refiners Association (2002) statistics. Thus, *per capita* intake of enzyme preparation TOS is calculated as roughly 1.2 μg/person/day, as follows: (0.104 kg sweetener/day) × (12 ppb (μg/kg) enzyme TOS in sweetener). Ingestion of enzyme preparation residues by a heavy consumer of starch-derived sweeteners will be somewhat higher than this value, since such individuals are expected to consume more than the per capita sweetener intake of 104 g/person/day. Although a factor of 10 is sometimes applied to the per capita intake of a substance to approximate consumption among heavy consumers, this would be highly exaggerative in the case of starch-derived sweeteners, which are distributed very broadly throughout the food supply. (Assuming consumption at 10-fold times the per capita would amount to more than 1000 g sweetener/person/day, one-third of the average daily diet.) Therefore, in this case a more appropriate factor would be three times the per capita intake, or 312 g sweetener/person/day. This results in an estimated intake of BD5088 enzyme preparation TOS of 3.6 μg/person/day. This high-consumer intake may be expressed as 0.00006 mg/kg bw/day for a 60-kg adult. By comparison, the NOAEL of 890 mg/kg bw/day observed in the 90-day toxicity study in rats is more than 14 *million* times the calculated high-consumer intake.

It is evident that an extremely wide margin of safety exists between the estimated daily intake and the NOAEL. Indeed, even if it is assumed that there is no removal of BD5088 α-amylase residues upon purification of the starch hydrolysis products, the amount of enzyme remaining in the finished sweetener could not exceed the amount added, 15 ppm (calculated as TOS), which would correspond to approximately 5 mg/person/day, or about 0.08 mg/kg bw/day, for heavy consumers. This level of exposure represents less than one ten-thousandth of the NOAEL.

In addition to its use in the production of starch-derived sweeteners, BD5088 α-amylase will also be used in early stages of the production of ethanol from corn or grain for use in alcoholic beverages. Like other α-amylase enzymes, BD5088 will be used to hydrolyze starch to provide an increased level of fermentable sugars, which may result in increased ethanol yields. When ethanol is produced by fermentation in this manner, it is purified through distillation. Because ethanol is relatively low boiling (78.5 °C), no measurable amount of

BD5088 α-amylase enzyme preparation will be present in the purified ethanol distillate.

The BD5088 α-amylase enzyme preparation is derived from controlled fermentation involving a non-pathogenic, non-toxic organism; it has been shown not to produce antibiotics, and it is processed from substances acceptable for general use or generally recognized as safe. The enzyme does not exhibit properties associated with known allergens in that it lacks sequence identity over eight contiguous amino acids and of 35% identity over 80 amino acids when compared to known allergens, and is readily degraded by pepsin digestion with a half-time of less than 0.3 min.

Based on current guidance (Pariza and Johnson, 2001), the safety of BD5088 α-amylase preparation for use in corn wet milling has been established. This safety evaluation requires an assessment of the toxigenic potential of the production strain. *Id.* In the pathogenicity study, mice were exposed to the spent growth medium (filter sterilized) in order to evaluate whether *P. fluorescens* synthesized any toxins which were active via the oral route. No toxigenic metabolites were present in the spent growth medium as indicated by the overall health of the mice during the course of the study. These results demonstrated that *P. fluorescens* biovar I, strain MB101, exerted no signs of pathogenicity or toxigenicity in Balb/c mice and does not represent an infectious threat to humans.

The *P. fluorescens* biovar I production strain was avirulent when administered orally to mice and is considered safe on this basis. The test article was free of transferable antibiotic resistance gene DNA based on the use of non-conjugative and poorly mobilizable plasmid vectors and the lack of detectable residual antibiotic resistance gene DNA in the test article. All introduced DNA was plasmid-based, fully sequenced, and determined not to contain any potentially toxigenic sequences. This plasmid DNA is well characterized and free of attributes that would render it unsafe for constructing microorganisms to be used in food grade products. The test article was free of antibiotic activity and contained no quantifiable antibiotics. The BD5088 enzyme preparation was free of oral toxins, based on the 13-week gavage study, in which there was no systemic toxicity. Respiratory inflammation was considered to be an artifact of the repeated oral gavage administration. Based on the entirety of the information discussed here, it may be concluded that BD5088 α-amylase preparation is safe for use as a processing aid for food produced by corn wet milling and in distilled alcohol production.

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