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Safety evaluation of a lipase enzyme preparation, expressed in *Pichia pastoris*, intended for use in the degumming of edible vegetable oil

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Abstract

BD16449 lipase is the product of a phospholipid-specific lipase gene expressed in the yeast *Pichia pastoris* strain DVSA-PLC-004. This type C phospholipid lipase (EC 3.1.4.3) is intended for use in the degumming of edible vegetable oil. BD16449 lipase was tested as a refined test article preparation (DV16449) for its effects on genotoxicity and in acute, inhalation, and subchronic toxicity studies. Dosages ranged from 5000 μg/plate for in vitro toxicity studies to 2000 mg/kg/day for in vivo toxicity studies. The highest oral dose tested in vivo (NOAEL of 2000 mg/kg/day) resulted in a safety margin of 133,000 based on the conservative estimate of the total human consumption of BD16449 lipase of 0.015 mg/kg/day. There was no toxicity reported for any of these studies including additional safety studies. A review of the literature indicates that *P. pastoris* fulfills recognized safety criteria pertinent to microbial production strains used in the manufacture of food enzyme preparations. The results of the toxicity studies presented herein attest to the safety of BD16449 lipase for use in the degumming of edible vegetable oil.

Keywords: Lipase; *Pichia pastoris*; Toxicology; Safety; Degumming

1. Introduction

Lipases are hydrolases, more specifically ester hydrolases, that release ester-linked side chains from glycerol lipids in the presence of water; for this reason, lipases are also referred to as glycerol ester hydrolases (EC 3.1.1.3). Lipases are ubiquitous in nature and can be found in bacteria, plants, and animals. They have been studied for almost 100 years (Jaeger and Eggert, 2002) and used safely in food processing applications for over 50 years (Pandey et al., 1999; Woolley and Petersen, 1994 and references therein). One of these applications is the use of lipases for the processing of vegetable oils.

Crude vegetable oil is composed primarily of triglycerides, molecules containing a fatty acid ester side chain attached to each carbon of the glycerol backbone (referred to as the SN1, SN2, and SN3 positions). Crude vegetable oil also contains phospholipids; phospholipids possess a phosphate ester at the SN3 position of the triglyceride instead of a fatty acid side chain. The major phospholipids found in vegetable oils like soybean, canola, and sunflower oils include phosphatidylcholine (PC) and phosphatidylethanolamine (PE); these oils also contain phosphatidylinositol (PI) and phosphatidic acid (PA). Together, these phospholipids represent the major source of phosphorus in crude vegetable oils. Phospholipids and other non-triglyceride components must be removed from the oil to ensure the proper flavor, stability, and appearance of the final oil.

The removal of phospholipids is accomplished using a refining step known as degumming (Hodgson, 1996). Degumming takes advantage of the amphiphilic nature of phospholipids; the presence of the polar, phospho-head group attached to the diglyceride, facilitates separation of phospholipids from the bulk oil in the presence of water.

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Upon addition of small amounts of water to crude oil, the phospholipids become hydrated and form a gum. This gum can then be separated from the oil by centrifugation. However, because the amphiphilic phospholipids are inherently good emulsifiers, they also trap triglyceride oil in the gum fraction resulting in oil yield losses during the degumming process. Phospholipid-specific lipases can be used to alter the emulsification properties of phospholipids and several phospholipid-specific lipases are currently used for this purpose in food processing (Clausen, 2001; Dahlke, 1998; Ulbrich-Hofmann, 2000). BD16449 lipase is a microbial phospholipid-specific lipase closely related to the well-characterized phospholipase from *Bacillus cereus* (Hergenrother and Martin, 2000). BD16449 lipase decreases the emulsifying properties of phospholipids by hydrolyzing PC and PE separating the phosphorus component from the glycerol backbone to produce 1,2-diacylglycerol (DAG) and the corresponding phosphate ester as represented in Fig. 1.

The use of BD16449 lipase during vegetable oil degumming improves oil yield both by reducing the amount of triglyceride oil lost during degumming and by producing 1,2-DAG oil from the phospholipid substrate as shown in Fig. 1. The specificity of cleavage classifies BD16449 lipase as a type C phospholipid-specific lipase (EC 3.1.4.3). Purified BD16449 lipase (including material purified from test article lot PLC-16449-PD267B) possesses a specific activity of approximately 2300 U/mg, where a unit is defined as the hydrolysis of 1 μmol phosphatidylcholine per minute at 37 °C, pH 7.3. The enzyme has a pH optimum of 7.5 and a temperature optimum of 60 °C. In addition, the enzyme possesses no measurable activity against a triglyceride lipase substrate. This paper describes DV16449, a preparation of BD16449 lipase that is expressed as a secreted, glycosylated protein in the yeast *Pichia pastoris*.

Using homologous recombination, the phospholipase C gene was directly integrated into the genomic *AOX1* site of a non-toxigenic and non-pathogenic host, *P. pastoris* strain SMD 1168 (Gleeson et al., 1998; Sreekrishna et al., 1997). The resulting production strain, DVSA-PLC-004 produces BD16449 lipase via a submerged, fed-batch, pure culture fermentation. The enzyme is secreted as a glycosylated protein with an apparent molecular weight of 34 kDa when analyzed by SDS–polyacrylamide gel electrophoresis (deglycosylated protein has a mass of 28.14 kDa when analyzed by mass spectroscopy).

Pichia pastoris strain DVSA-PLC-004 was used to produce BD16449 lipase test article, designated as DV16449 (lot PLC-16449-PD267B), for the safety and toxicology testing presented in this paper.

2. Materials and methods

2.1. Test article preparation

The *P. pastoris* strain SMD1168, a commercially available protease-deficient derivative of the wild-type strain NRRL Y-11430 (ATCC Accession 76273) (Gleeson et al., 1998; ATCC, 2005) was transformed with the gene for BD16449 lipase. Individual transformants were screened for maximum expression of active BD16449 lipase protein. This screen resulted in the selection of the production strain designated as DVSA-PLC-004.

The BD16449 lipase test article was produced from strain DVSA-PLC-004 in a fermentation and recovery pilot plant in accordance with current good manufacturing practices (cGMP) for foods. The pilot plant process is representative of the manufacturing process for the commercial enzyme, at scale.

The production strain DVSA-PLC-004 was cultured from a frozen glycerol stock in successive seed flask stages and ultimately in 30 and 500 L fed-batch, pure culture fermentations. The batch and feed media, and process and quality control systems were representative of those specified in the manufacturing process. For production of test article DV16449, the recovery steps used in the manufacturing process were scaled-down to equivalent unit operations in the pilot plant.

The fermentation broth was centrifuged to remove most of the initial cell mass (>99%). The process stream was then clarified using a plate and frame filter press to remove any remaining cells followed by an ultrafiltration step. The test article material was lyophilized to increase shelf life. This material was designated as lot PLC-16449-PD267B and contains 315 U PLC activity per milligram dry weight. The material was stored at 4–8 °C in sterile PETG bottles sealed under nitrogen. The Sponsor always supplied the test article (DV16449) to external study sites.

Prior to use in toxicological studies, DV16449 (lot PLC-16449-PD267B) was analyzed for chemical and microbial composition, including the absence of the production strain. The results demonstrated that the samples conformed to the microbial and chemical specifications established for enzyme preparations used in food processing, as published in the Food Chemical Codex (FCC), Fifth Edition, 2004 (Committee on Food Chemicals Codex, 2004), and by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (FAO/WHO, 2001). The test article samples were also tested for and shown to be free of antimicrobial activity and mycotoxins. Tests were performed to assess antimicrobial/antibiotic activity in accordance with JECFA's procedure: Determination of Antimicrobial Activity (FAO/WHO, 1992). Mycotoxin activity was analyzed using HPLC (high performance liquid chromatography) for aflatoxins and ochratoxin A and TLC (thin layer chromatography) for T-2 toxin and sterigmatocystin. The limits of detection (LOD) for the mycotoxins tested were as follows: aflatoxin B1 (1.0 ppb), aflatoxin B2 (1.0 ppb), aflatoxin G1 (1.0 ppb), aflatoxin G2 (1.0 ppb), ochratoxin A (2 ppb), T-2 toxin (0.1 ppm), and sterigmatocystin (200 ppb).

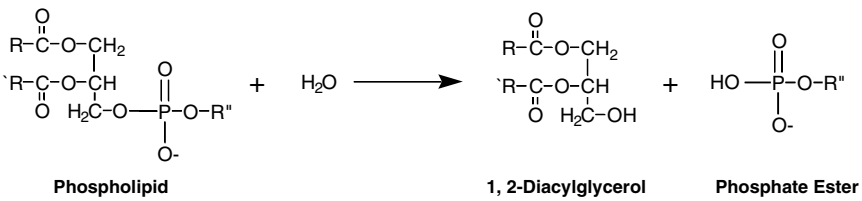

Fig. 1. Diagram showing the hydrolysis reaction carried out by lipase BD16449. In the figure, R and R′ represent the fatty acid acyl chains and R″ represents the head group of the phospholipid (for example, choline when the phospholipid is phosphatidylcholine).

3. Results

3.1. Genotoxicity studies

3.1.1. Mutagenicity assay

The objective of this study (Ames et al., 1975) was to evaluate the test article, DV16449, for the ability to induce reverse mutations at the histidine locus in four tester strains of *Salmonella typhimurium* (TA98, TA100, TA1535, and TA1537), and at the tryptophan locus in *Escherichia coli* tester strain WP2*uvr*A(pKM101), in the presence and absence of an exogenous metabolic activation system (S9). The tester strains were exposed to the test article using the treat and plate modification.

Doses tested in the mutagenicity assay were selected based on the results of a dose range finding assay using tester strains TA100 and WP2*uvr*A(pKM101) and 10 doses of test article ranging from 10.3 to 7690 μg of test article per mL of treat and plate reaction mixture, one plate per dose, both in the presence and absence of S9 mix.

Tester strains used in the mutagenicity assay were *S. typhimurium* tester strains TA98, TA100, TA1535, and TA1537 and *E. coli* tester strain WP2*uvr*A(pKM101). The assay was conducted in both the presence and absence of S9 mix along with concurrent vehicle and positive controls using three plates per dose. The doses tested in the mutagenicity assay with the *Salmonella* tester strains in both the presence and absence of S9 mix were 7690, 5120, 1540, 512, and 154 μg of test article per mL of treat and plate reaction mixture (2500 μL of S9 mix or buffer, 500 μL of appropriate tester strain, and 250 μL of vehicle or test article dose). These doses are equivalent to the following doses tested using the standard plate incorporation exposure method (5000, 3330, 1000, 333, and 100 μg/plate). The doses tested in the mutagenicity assay with WP2*uvr*A(pKM101) were 7690, 5120, 1540, 512, 154, 51.2, and 15.4 μg of test article per mL of treat and plate reaction mixture (2500 μL of S9 mix or buffer, 500 μL of appropriate tester strain, and 250 μL of vehicle or test article dose). These doses are equivalent to the following doses tested using the standard plate incorporation exposure method (5000, 3330, 1000, 333, 100, 33.3, and 10.0 μg/plate). Results of the initial mutagenicity assay were confirmed in an independent experiment.

For all replicate platings, the mean revertants per plate and the standard deviation were calculated.

The results of the *Salmonella–E. coli*/mammalian-microsome reverse mutation assay with a confirmatory assay treat and plate method indicated that under the conditions of this study, DV16449 did not cause a positive increase in the mean number of revertants per plate with any of the tester strains in the presence or absence of Aroclor™ induced rat liver (S9).

3.1.2. Chromosomal aberrations in cultured human peripheral blood lymphocytes assay

The objective of this in vitro assay was to evaluate the ability of DV16449 to cause structural chromosomal aberrations in cultured human lymphocytes with and without an exogenous metabolic activation system (Evans, 1962, 1976).

Cell culture grade water was the vehicle of choice for this study. The highest dose tested in the assay, 5000 μg/mL, is the high dose recommended by the OECD Testing Guidelines. The stock solution and its dilutions were dosed using a dosing volume of 10% (100 μL/mL), and the vehicle control cultures were treated with 100 μL/mL of cell culture grade water.

In the initial chromosomal aberrations assay, the treatment period was for 3 h with and without metabolic activation, and cultures were harvested ~22 h from the initiation of treatment. Replicate cultures of human whole blood lymphocytes were incubated with test article at 33.9, 48.4, 69.2, 98.9, 141, 202, 288, 412, 588, 840, 1200, 1720, 2450, 3500, and 5000 μg/mL with and without metabolic activation. Cultures treated with concentrations of 412, 1200, 2450, and 5000 μg/mL without metabolic activation and 588, 1200, 2450, and 5000 μg/mL with metabolic activation were analyzed for chromosomal aberrations. No significant increase in cells with chromosomal aberrations, polyploidy, or endoreduplication was observed in the cultures analyzed.

In the confirmatory chromosomal aberrations assay, the treatment period was ~22 h without metabolic activation and 3 h with metabolic activation, and cultures were harvested ~22 h from the initiation of treatment. Replicate cultures of human whole blood lymphocytes were incubated with test article at 62.5, 125, 250, 500, 1000, 2000, 3000, 4000, and 5000 μg/mL without metabolic activation and 1000, 2000, 3000, 4000, and 5000 μg/mL with metabolic activation. Cultures treated with concentrations of 2000, 3000, 4000, and 5000 μg/mL without and with metabolic activation were analyzed for chromosomal aberrations. No statistically significant increase in cells with chromosomal aberrations, polyploidy, or endoreduplication was observed in the cultures analyzed.

Statistical analysis employed the Cochran–Armitage test for linear trend and Fisher's Exact Test (Thakur et al., 1985) to compare the percentage of cells with aberrations in treated cells to the results obtained for the vehicle controls.

DV16449 was considered negative for inducing structural and numerical chromosomal aberrations in cultured human peripheral blood lymphocytes without and with an exogenous metabolic activation system.

3.1.3. Mouse micronucleus assay

The objective of this study was to evaluate the test article, DV16449, for in vivo clastogenic activity and/or disruption of the mitotic apparatus by detecting micronuclei in polychromatic erythrocytes (PCE) in CD-1® (ICR)BR mouse bone marrow (Heddle et al., 1983).

In the dose range finding assay, the test article was formulated in sterile water for injection and administered once, by oral gavage to three males and three females per

Table 1
Administration of test article in micronucleus assay

Target dose level (mg/kg)	Stock concentration (mg/mL)	Dosing volume (mL/kg)	Route of administration	Animals/harvest	Timepoint[a]
				24 h Male	48 h Male
Positive control, 80	8	10	Oral gavage	6	—
Vehicle control, 0	0	10	Oral gavage	6	6
500	50	10	Oral gavage	6	—
1000	100	10	Oral gavage	6	—
2000	200	10	Oral gavage	6	6

Vehicle control = sterile water for injection, positive control = cyclophosphamide.
[a] Six animals were dosed to ensure the availability of five animals/harvest timepoint for analysis.

dose level. The animals were dosed at 500, 1000, or 2000 mg/kg and observed for up to 2 days after dosing for toxic signs and/or mortality.

Data analysis was performed using an analysis of variance (Winer, 1971) on untransformed proportions of cells with micronuclei per animal on untransformed PCE:NCE ratios when the variances were homogeneous. Ranked proportions were used for heterogeneous variances. If the analysis of variance was statistically significant, Dunnett's *t* test (Dunnett, 1955, 1964) was used to determine which dose groups, if any, were statistically significantly different from the control. Analyses were performed separately for each sampling time.

Based on the results of the dose range finding assay, the high dose chosen was 2000 mg/kg, the limit dose based on regulatory guidelines. In the micronucleus assay, the test article was formulated in sterile water for injection and administered once as indicated in Table 1.

Bone marrow was extracted and at least 2000 PCEs per animal were analyzed for the frequency of micronuclei. Cytotoxicity was assessed by scoring the number of PCEs and normochromatic erythrocytes (NCEs) in at least the first 500 total erythrocytes for each animal.

The test article, DV16449, did not induce signs of clinical toxicity in the animals treated at dose levels up to 2000 mg/kg (the limit dose based on regulatory guidelines). DV16449 did not induce statistically significant increases in micronucleated PCEs at any test article dose examined (500, 1000, and 2000 mg/kg). In addition, DV16449 was not cytotoxic to the bone marrow (i.e., no statistically significant decrease in the PCE:NCE ratio) at any dose of the test article.

The test article, DV16449, was evaluated as negative in the mouse bone marrow micronucleus assay under the conditions of this assay.

3.2. Acute toxicity study

3.2.1. Acute oral toxicity study in rats

The purpose of this study was to determine the acute toxicity of DV16449 when administered to rats as a single dose by oral gavage (Gad, 1995; Speid et al., 1990). The test article, DV16449, was supplied by the Sponsor as a lyophilized powder. The test article was then prepared into dosing solutions for oral administration via gavage. Initially, one experimentally naïve female Sprague–Dawley rat was dosed at 2000 mg/kg. Based on the results for this animal, four additional experimentally naïve female Sprague–Dawley rats were dosed at 2000 mg/kg. Mortality and clinical observations were evaluated daily. Body weights were recorded weekly. All surviving animals were sacrificed on Day 15 and a gross necropsy was performed.

Mortality was not observed in any of the animals at 2000 mg/kg. No clinical signs were observed in the animals during the study. No effect was seen on body weight in the animals for Days 8 and 15. No visible lesions were observed in any of the animals at necropsy.

Since all five animals survived, testing was terminated and the oral LD_{50} for DV16449 was considered greater than 2000 mg/kg.

3.3. Subchronic toxicity study

3.3.1. Ninety day oral toxicity study in rats

The purpose of this study was to evaluate the toxicity of DV16449 when administered orally, via gavage, once daily for 90 consecutive days to Sprague–Dawley rats (Gad, 1995; Speid et al., 1990).

The test article, DV16449, was supplied by the Sponsor as a lyophilized powder. The test article was then prepared into dosing solutions for oral administration via gavage. One hundred and sixty experimentally naïve Sprague–Dawley rats (80 males and 80 females), approximately 6 weeks old and weighing 169–204 g for males and 133–172 g for females at the outset of the study were assigned to treatment groups as shown in Table 2.

Data were evaluated using LABCAT Body Weight module version 4.65, LABCAT Hematology module version 4.3, LABCAT Clinical Chemistry module version 4.3 and LABCAT Organ Weights/Necropsy module version 3.28. In addition, SYSTAT version 9.01, developed by SPSS, Inc., was used in body weight and food consumption data analysis.

Body weight, food consumption, hematology, coagulation, clinical chemistry, and organ weight data were evaluated. The evaluation of the equality of means was made by a one-way analysis of variance using the *F* distribution to assess statistical significance. If statistically significant differences between the means were found, Dunnett's test was used to determine the degree of significance from the control means ($p < 0.05$ and $p < 0.01$).

Table 2
Treatment groups in subchronic toxicity study

Group	Dose level[a] (mg/kg/day)	Concentration (mg/mL)	Dose volume (mL/kg)	Number of animals	
				Male	Female
1. Control (vehicle)	0	0	10.0	20	20
2. DV16449 low-dose	500	50	10.0	20	20
3. DV16449 mid-dose	1000	100	10.0	20	20
4. DV16449 high-dose	2000	200	10.0	20	20

[a] Doses tested were selected based on the results of a dose range finding study.

Animals were dosed once daily for 90 days. Mortality and clinical observations were evaluated daily. Body weights and food consumption were recorded weekly. Ophthalmology examinations were performed prior to treatment initiation and during the last week of treatment. Blood for evaluation of hematology, coagulation, and clinical chemistry was collected on Days 91 or 92. All surviving animals were sacrificed on Days 91 or 92. Selected tissues were harvested at necropsy, selected organs weighed, and selected tissues from the control and high dose groups evaluated microscopically.

There were three animals found dead on the study. All were considered related to gavage incidents.

There were no test article-related clinical signs of toxicity noted.

All test article-treated males had decreased body weight gains during the first week of the study. This resulted in decreased group mean body weights compared to the concurrent controls on Day 8 for males at all dose levels. During the second week of the study, weight gains were increased for all test article-treated males compared to controls and therefore, group mean body weights were comparable to controls in all groups by Day 15. Decreased group mean weight gains were also noted for males in the 1000 and 2000 mg/kg dose groups on Day 43 and the 2000 mg/kg dose group on Day 50. These later changes did not result in differences in group mean body weights compared to the controls.

Group mean weight gains were increased for the 2000 mg/kg females on Day 15, the 1000 mg/kg females on Day 29 and decreased for the 1000 mg/kg females on Day 22. These changes had no effects on group mean body weight for the females.

Group mean food consumption was increased for the 1000 mg/kg males on Day 8. Group mean food consumption was decreased for the 1000 and 2000 mg/kg males on Day 15 and the 2000 mg/kg males on Days 36 and 57.

A statistically significant increased group mean food consumption was noted for the 500 mg/kg females on Day 15 and the 1000 mg/kg females on Day 22. However, as no dose–response was noted, these changes were considered incidental and unrelated to treatment.

There were no test article-related ophthalmological findings noted during the study. There were no test article-related changes in hematology, coagulation or clinical chemistry parameters or erythrocyte morphology.

There were no test article-related gross necropsy findings. There were no test article-related changes in absolute or relative-to-body or relative-to-brain organ weights. Definitive test article-related lesions were not noted in this study.

In conclusion, oral administration of DV16449 at doses of 500, 1000 or 2000 mg/kg/day, via oral gavage, once daily for 90 consecutive days resulted in only minimal, transient changes in group mean body weights, weight gains, and food consumption. Based on these findings, the no-observed-adverse-effect level (NOAEL) is considered to be greater than 2000 mg/kg/day.

3.4. Inhalation toxicity study

3.4.1. Acute, nose-only, inhalation toxicity study in rats

The six-hour nose-only inhalation toxicity of DV16449 was evaluated in Sprague–Dawley rats (OECD, 1981; US EPA, 1998). A limit test was performed in which a group of five male and five female rats received a single six-hour nose-only inhalation exposure to a time-weighted average aerosol concentration (gravimetrically determined) of 2.34 mg/L. The mass median aerodynamic diameter and geometric standard deviation of the sampled particles were $3.6\ \mu \pm 2.68$. The percentage of particles $\leqslant 4.0\ \mu$ was determined to be 55%. Following the exposure, the limit test rats were observed daily and weighed weekly. A gross necropsy examination was performed on all limit test animals at the time of scheduled euthanasia (Day 14.)

No mortality occurred during the limit test. The most notable clinical abnormalities observed during the study included transient incidences of few feces, breathing abnormalities, and dark material around the facial area which are findings consistent with dosing an inhalation study. Slight body weight loss was noted for two female animals during the study Day 0–7 body weight interval. Body weight gain was noted for all other animals during the test period. All animals exceeded their initial body weight by study termination (Day 14). No significant gross internal findings were observed at the time of scheduled necropsy on study Day 14.

Under the conditions of this test, the acute inhalation LC50 of DV16449 was estimated to be greater than 2.34 mg/L in the rat.

3.5. Additional safety studies

3.5.1. Primary eye irritation in rabbits

The purpose of this study was to determine the potential irritant and/or corrosive effects of the test article on eyes of

rabbits (Draize, 1959). A 2.0% solution of the test article, DV16449, was prepared and was instilled into the conjunctival sac of the right eye of each of six female New Zealand White rabbits at a volume of 0.1 mL/eye. Upon instillation, each eye was held closed for ~1 s to prevent loss of material. The left eye was untreated and served as the control. Both eyes of all animals were examined and scored for ocular irritation prior to dose, 1 h after dose and at 24, 48, and 72 h post dose.

No ocular irritation involving the cornea or iris was observed in the test-treated eyes during the study. Non-positive conjunctival redness was observed in 6 of 6 animals at 1 h and non-positive conjunctival chemosis and discharge was observed in 1 of 6 animals at 1 h. No signs of irritation were observed in any of the animals at 24, 48 or 72 h post treatment.

Under the conditions of this study, a 2.0% solution of DV16449, was found to be a minimal irritant (Class 3) in rabbits.

3.5.2. Primary dermal irritation in rabbits

The purpose of this study was to determine the potential irritant and/or corrosive effects of the test substance on the skin of rabbits. A 2.0% solution of the test article, DV16449, was prepared and was applied at 0.5 mL/site to one site on the clipped dorsal trunk of three female New Zealand White rabbits. The exposure period was 4 h. Observations for topical irritation and corrosivity were recorded for each site predose, immediately post unwrap and at 24, 48 and 72 h after unwrap (Table 3). Grading of irritation was according to the method of Draize (1959).

Under the conditions of this study, a 2.0% solution of DV16449, was found to be non-irritating in rabbits (PII = 0.00).

3.5.3. Delayed contact hypersensitivity in guinea pigs

The purpose of this study was to determine if a test article elicits a delayed dermal contact hypersensitivity response in guinea pigs by the measurement of skin reactivity (Magnusson and Kligman, 1969). For the induction phase of this study, 20 guinea pigs (10/sex) in the test article group were induced with three six-hour occluded dermal applications of DV16449 at 2.0% in sterile water for injection. A vehicle group of 10 animals (5/sex) was induced in the same manner with sterile water for injection. A positive control group of six animals (3/sex) was induced with a known dermal sensitizer: 1-chloro-2,4-dinitrobenzene (DNCB) in 80% ethanol.

Fourteen days after the last induction, all animals were dermally challenged with occluded applications at naïve test sites. Animals in the test article and vehicle control group were challenged with DV16449 at 2.0% in sterile water for injection. Animals in the positive control group were challenged with DNCB (0.2% in acetone). On the day following the challenge, all animals were depilated and approximately 4 h later were scored for dermal irritation (24 h). Scoring was repeated at 48 h. Table 4 shows that, under the conditions of this study, induction with DV16449 at 2.0% did not elicit a delayed contact hypersensitivity response in guinea pigs when challenged with the test article at 2.0%.

Table 3
Observation periods and dermal observation

Observation period	Dermal observation
Immediate, 24, 48, and 72 h	No erythema or edema

4. Discussion

Pichia pastoris methylotrophic yeast strain DVSA-PLC-004 is the production strain for BD16449 lipase and was also used to produce BD16449 lipase test article, DV16449.

Assessment of the safety of the production strain was conducted according to the safety guidelines of Pariza and Foster (1983), which state that a non-toxigenic organism is "one which does not produce injurious substances at levels that are detectable or demonstrably harmful under ordinary conditions of use or exposure." Upon evaluating various safety considerations (International Food Biotechnology Council (IFBC), 1990; OECD, 1992; Pariza and Foster, 1983; Pariza and Johnson, 2001) and the potential modes of toxicity such as allergenicity and the production of mycotoxins or antibiotics, *P. pastoris* fulfills the criteria of a non-toxigenic and non-pathogenic microorganism.

Extensive reviews of literature databases failed to generate documentation of toxigenic effects of *P. pastoris*. Yeasts are not known to produce toxins that are active by the oral route (Pariza and Johnson, 2001) and *P. pastoris* has been classified as Biosafety Level 1 (BL-1), a category reserved for well-characterized agents not known to cause disease

Table 4
Summary of challenge results

Group	Challenge	Percentage of animals with scores after 24 and 48 h									
		24-hour scores					48-hour scores				
		0	±	1	2	3	0	±	1	2	3
Vehicle	Sterile water for injection	100	0	0	0	0	100	0	0	0	0
	DV16499 at 2.0%	100	0	0	0	0	100	0	0	0	0
Test article	DV16499 at 2.0%	100	0	0	0	0	100	0	0	0	0
Positive control	DNCB (0.2% in acetone)	0	0	0	50	50	0	0	0	40	60

in healthy human adults and requiring minimal safety precautions in handling and storage (CDC, 1999).

Pichia pastoris has been safely used for the production of over 300 recombinant proteins since the mid-1980s (Cereghino and Cregg, 2000) and fulfills the criteria of several safety evaluations. Many human genes have been expressed in *P. pastoris* for pharmaceutical use. Two such recombinant proteins, Angiostatin and Endostatin, have completed Phase I clinical trials and are entering into Phase II clinical trials (Herbst et al., 2002). In addition, *P. pastoris* itself is approved by FDA as a source of animal feed protein for use in broiler feed up to 10% of the total feed (FDA, 1993). Toxicity studies done in support of the above-referenced *P. pastoris*-approved animal feed (including a pathogenicity study in mice, an acute oral toxicity study in rats, a subacute oral toxicity study in rats, and a two generation teratology study in rats) also demonstrated—per FDA's review in 1993—that *P. pastoris* is neither pathogenic nor toxigenic (FDA, 1993).

To evaluate the safety of production strain DVSA-PLC-004 for the heterologous expression of a food grade enzyme, refined test article, DV16449 (representative of the final product) was used in genotoxicity studies and in acute, inhalation, and subchronic toxicity studies. There was no genotoxicity reported in the mutagenicity assay, the chromosomal aberration assay, or the mouse micronucleus assay. There was no in vivo toxicity reported in the acute, inhalation, and subchronic toxicity studies. Additional dermal and eye irritation safety studies were also devoid of any relevant toxicity.

The NOAEL from the subchronic toxicity study can be used in the calculation of the safety margin based on the use level of the enzyme in the application and human consumption data. The safety margin is calculated by dividing the NOAEL from the subchronic study by the maximum estimated daily intake (EDI).

The NOAEL or highest dose level fed to rats in the 90-day oral toxicity study was 2000 mg/kg/day (10 mL of a 200 mg/mL solution) which corresponds to 630,000 U of enzyme/kg/day (2000 mg × 315 U/mg) or 1680 mg TOS[1]/kg/day. The percent TOS for DV16449 is 84%.

When the enzyme is applied in the oil degumming process, the enzyme is inactivated and removed by the process. However, to provide a "worst case" scenario for the calculation of possible human exposure, an assumption is made that all of the enzyme added to the crude oil is retained in the final vegetable oil. The highest recommended use rate of the enzyme (10 ppm) would result in a maximum EDI of 0.015 mg enzyme/kg/day or 0.0126 mg TOS/kg/day assuming the average per capita daily consumption of vegetable oils and fats for an average person, (i.e., weighing 60 kg) is 90 g/person/day (USDA, 2005). [2]

Thus, the safety margin calculated with or without TOS would be:

$$\frac{2000\ \text{mg/kg/day}}{0.015\ \ \text{mg/kg/day}} = 13.3 \times 10^4$$

OR

$$\frac{1680\ \text{mg TOS/kg/day}}{0.0126\ \text{mg TOS/kg/day}} = 13.3 \times 10^4.$$

5. Conclusion

The safety evaluation, of the test article DV16449, clearly demonstrates that BD16449 lipase product:

(1) is derived from a non-toxigenic and non-pathogenic production strain (DVSA-PLC-004) which has a history of safe use,
(2) is derived from a controlled, pure fermentation process,
(3) does not induce any in vitro genotoxicity or in vivo toxicity in rats when inhaled, or consumed, at levels up to 2000 mg/kg,
(4) has a safety margin of 133,000 based on the ratio of the NOAEL of 2000 mg/kg/day to the conservative estimate of total human consumption of BD16449 lipase of 0.015 mg/kg/day or 0.0126 mg/kg/day on a TOS basis.

Thus, it is concluded that the documented safety record of *Pichia pastoris* and the results of toxicology studies described here for test article DV16449, specifically, and in the published literature for lipases in general, indicate that BD16449 lipase is safe for its intended use in the degumming of edible vegetable oil.

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[1] The Pariza and Foster (1983) paper recommends the use of the total organic solids (TOS) concept when determining a reliable estimate of enzyme use and consumption. TOS is defined as the sum of the organic compounds, excluding diluents, contained in the final enzyme preparation. TOS (%) = 100 − % ash − % water − % diluent.

[2] Please note the figure, 90 g/person/day, was derived from the best available data supplied by USDA. The USDA-ERS consumption table for added fats and oils lists several sources of vegetable oils and fats as well total vegetable oil and fat consumption per capita on a total fat content basis. Therefore, the most conservative estimate of 90 g/person/day was calculated from the 2003 per capita consumption of 73.1 £ of vegetable derived added oils and fats on a total fat content basis.

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