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Safety Evaluation of β-Glucanase Derived from *Trichoderma reesei*: Summary of Toxicological Data

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Abstract—Barlican, a β-glucanase enzyme obtained from *Trichoderma reesei*, was produced by a fermentation process and subjected to a series of toxicological tests to document its safety for use as a feed additive. The enzyme product was examined for general oral toxicity, inhalation toxicity, irritation to eye and skin, skin sensitization and mutagenic potential. An extensive literature search on the production organism was also conducted. Furthermore, safety for target species was assessed in a 28-day oral toxicity study with broilers. A strong skin-sensitizing potential of the β-glucanase enzyme was detected, but no other evidence of oral or inhalation toxicity, mutagenic potential, eye or skin irritancy was found. Feeding of the β-glucanase enzyme at dietary levels up to 10,000 ppm in the 90-day subchronic toxicity study in rats did not induce noticeable signs of toxicity. In addition, no adverse effects were observed when broiler chicks were fed dietary concentrations of the β-glucanase enzyme up to eight times the daily recommended dose. It is therefore concluded that this β-glucanase preparation is safe for use in feed of the intended target species. However, some occupational health precautions should be taken to avoid skin contact and inhalation, as is the case for almost all enzyme proteins.

INTRODUCTION

There is a long history of the safe use of β-glucanases in the food processing industry. Barlican is a β-glucanase enzyme preparation produced by controlled submerged fermentation of a selected pure culture of the fungus *Trichoderma reesei*. The principal activity of the product is due to endo-1,3(4)-β-glucanase, which is able to hydrolyse polysaccharides such as barley β-glucans. The product also includes other active carbohydrates, such as hemicellulase and cellulases. The powdered product is standardized with wheat flour at an enzyme activity of at least 8000 β-glucanase units (BGU)/g, while the liquid product, Barlican 25% L, is standardized and stabilized by glycerol at an enzyme activity of at least 2000 BGU/g. This liquid can be used for post-pelleting application.

Barlican has been developed especially to improve the nutritional value of barley in poultry diets by alleviating the antinutritional properties of the β-glucans. These polysaccharides are known to give rise to highly viscous conditions in the small intestine of barley-fed chicks, which can adversely affect nutrient utilization. This problem can be overcome by inclusion of Barlican at a standard β-glucanase activity.

The recommended doses for barley-based poultry diets are 100 ppm (800 BGU/kg diet) for the powdered product and 400 ppm (800 BGU/kg diet) for the liquid product.

The β-glucanases from *Disporotrichum dimorphosporum* and from *Penicillium emersonii* are used in the UK and France for several applications in beverages and starch (AMFEP, 1992). Published data on the safety of cellulases from *Trichoderma reesei* shows no harmful effects (Hjortkjaer *et al.*, 1986). In this review we summarize studies conducted to establish the safety of Barlican when used as a feed additive in poultry feed. The studies were designed to elucidate target species, farmer and industrial health effects.

The enzyme product was examined for general oral toxicity (acute and subchronic), inhalation toxicity, irritation to eye and skin, skin sensitization and mutagenic potential. An extensive literature search on the safety aspects of the production organism has been conducted (Nevalainen, 1994). This review shows that *T. reesei* can be regarded as non-pathogenic and as a safe production organism for enzymes used in food processing and other industrial applications. In addition, we tested our production organism for the production of toxins and antibiotics. Furthermore, safety for target species was assessed in

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Abbreviations: BGU = β-glucanase unit; FGR = feed conversion efficiency; ILOB = TNO Institute of Animal Nutrition and Physiology; NOEL = no-observed-effect level; OECD = Organisation for Economic Cooperation and Development; TOS = total organic solids.

a 28-day oral toxicity study with broiler chicks. The studies were undertaken in the period 1993–1994.

MATERIALS AND METHODS

The test material (referred to as the 'tox-batch') was produced by the procedure used for the commercial preparation of Barlican. The production process is performed according to the requirements of ISO9002 and includes the fermentation process, recovery (downstream processing) and formulation of the product. The purification process was followed by spray-drying to produce the final, non-standardized tox-batch. The tox-batch was used in all toxicity studies except the target species safety study where the standardized, commercial preparation was used. In Table 1, results of the characterization of the tox-batch by chemical and microbial analysis are presented (van der Lecq, 1994b). The stability of the tox-batch during the period of investigation was confirmed by analysis of the enzyme activity. The initial enzyme activity of the tox-batch is approximately 26,800 BGU/g with a Total Organic Solids (TOS) value of 93.5%.

The selection of the tests and the methods used in the individual tests were based on the guidelines of the Organisation for Economic Cooperation and Development (OECD, 1984) and EEC (EEC, 1992b) and were in accordance with the toxicological principles of the EC guidelines for the assessment of additives in animal nutrition (Dir 94/40/EC); studies concerning the safety of the use of the additive (EEC, 1994) were as follows:

Bacterial gene mutation, Ames test:	OECD 471 and EEC 67/548/EEC B14
Chromosome aberration *in vitro*:	OECD 473 and EEC 67/548/EEC B10
Acute oral toxicity in rat:	OECD 401 and EEC 84/449/EEC B1
Acute inhalation toxicity in rat:	OECD 403 and EEC 92/69/EEC B2
Skin irritation in rabbit:	OECD 404 and EEC 92/69/EEC B4
Eye irritation in rabbit:	OECD 405 and EEC 92/69/EEC B5
Skin sensitization in guinea pig:	OECD 406 and EEC 92/69/EEC B6
14-Day rangefinding dietary toxicity:	OECD 407 and EEC 92/69/EEC B7
90-Day oral dietary toxicity in rat:	OECD 408 and EEC 87/302/EEC B
Antibiotic production:	AMFEP: JECFA method FNP 19 (JECFA, 1981)
28-Day oral toxicity in broiler chicks:	EC DIR 94/40/EC.

All animals were subjected to daily clinical observation. Body weight and feed consumption were measured every 2 wk and on the day before autopsy. Feed and water consumption was determined during a period of 4 days after 14 days of treatment (age 19–23 days) per cage. Feed conversion efficiency was determined per cage after 14 and 28 days of treatment. On day 29 of the experimental period, prior to post-mortem examination, all animals were fasted for approximately 2 hr. Subsequently, blood samples were collected from the wing-vein of 24 broilers (four per cage) of the control group and 12 broilers (two per cage) of the highest treatment group for clinical laboratory investigations. The parameters assessed are listed in Table 2. Furthermore, macroscopic observations and organ weights were recorded from all broilers used for blood sampling (Table 3). From all intercurrent deaths and at scheduled termination from the animals used for blood sampling and from 12 animals (two per cage) from the intermediate groups, samples from the following tissues were collected and fixed: heart, kidneys, liver, spleen and all gross lesions from animals undergoing autopsy procedures. A histopathological examination was performed on the collected organs of the animals used for blood sampling only.

There were no changes in clinical appearance, weight gain, feed conversion efficiency, clinical laboratory investigations, macroscopic examination, organ weights and microscopic examination that were considered to be an effect of treatment. It was concluded that no evidence of toxicity was observed in broilers treated for 28 days at a dose level of 800 ppm Barlican in the diet (Schoenmakers, 1994a; Schutte and de Jong, 1994).

RESULTS

Toxicological studies on target species

28-Day oral toxicity in broiler chicks (diet). The target species safety of Barlican was examined in broiler chicks (Ross) housed in cages during a 28-day feeding study (5–33 days of age). Four groups each consisting of 90 chicks received Barlican in the basal diet (composition: 60% cereals; 20% soybean oilmeal) at levels of 0 (control), 100, 400 and 800 ppm. The dietary efficacy study was performed at the ILOB–TNO facilities; additional parameters for the target species safety were analysed at a contract laboratory in The Netherlands.

Oral toxicity studies

Acute oral toxicity in rats. The tox-batch was suspended in purified water and administered once orally by gavage, to five rats of each sex, at 2000 mg/kg body weight. Animals were subjected to daily observation and weekly determination of body

Table 1. Analytical results of β-glucanase enzyme preparation from *Trichoderma reesei* containing tox-batch*

Parameter	Result
Identity:	
Appearance	
Description	Lump-free powder
Colour	Creamish
Odour	Typical, not bad
Identification (SDS–PAGE)	Conforms
Identification (Native–PAGE)	Conforms
Foreign matter	Absent
Particle size	100% < 0.4 mm
Enzymatic assay	
β-Glucanase (new definition)	26,810 BGU†/g
Material balance	
Dry matter	95.8%
Total protein (Kjeidah × 6.25)	54.8%
Total carbohydrates	10.0% (rec. 90%)
Total organic solids (TOS)‡	93.5%
Ashes	2.3%
Residual minerals	
Heavy metals (as Pb)	<40 ppm
Pb	<0.08 ppm
Cd	0.01 ppm
As	0.12 ppm
Hg	<0.04 ppm
K	3250 ppm
Na	465 ppm
Ca	2140 ppm
Mg	570 ppm
pH (5% solution)	5.5
Toxins	
Aflatoxin B_1	<10 ppb
Ochratoxin	<40 ppb
T2 toxin	<200 ppb
Zearalenone	<200 ppb
Antimicrobial activity	Negative by test
Microbiological determinations	
Total viable count	60 CFU/g
Moulds	<10 CFU/g
Enterobacteriaceae	<10 CFU/g
Salmonellae	Absent in 25 g
Escherichia coli	Absent in 25 g
Staphylococcus aureus	Absent in 1 g
Clostridium perfringens	Absent in 1 g
Pseudomonas aeruginosa	Absent in 1 g
Production strain	Absent in 15 g

CFU = colony-forming unit

*From van der Lecq (1994b).

†One β-glucanase unit (BGU) is defined as the amount of enzyme which liberates 0.278 μmol reducing sugars measured as glucose equivalents per minute under the conditions of the test.

‡Total Organic Solids (TOS) is defined as 100% − (A + W + D)% where A is the ash content, W is the water content and D is the diluent content. TOS was 93.5% in the tox-batch.

weight. Macroscopic examination was performed at the end of the 14-day experimental period.

No deaths and no treatment-related clinical signs were observed during the study. The body weight gain shown by the animals over the study period was similar to that expected in normal untreated animals of the same age and strain. Macroscopic post-mortem examination of the animals at termination did not reveal significant abnormalities. The oral LD_{50} value of the tox-batch in rats of either sex was established as exceeding 2000 mg/kg body weight (Daamen, 1994a).

Subacute 14-day oral toxicity (range-finding) in rats. The tox-batch was examined in a 14-day study with four groups of five male and five female young Specified Pathogen Free (SPF)-bred Wistar rats, which received the test substance in the diet at levels of 0 (control), 1000, 5000 and 20,000 ppm.

Test substance preparations in the diet appeared to be stable and homogeneous and sufficiently accurate concentrations were encountered for the purpose of this study. There were no changes in clinical appearance, body weights, food consumption, clinical laboratory investigations, macroscopic examination, organ weights and microscopic examination considered to be related to treatment. It was concluded that no evidence of toxicity was observed in rats treated for 14 days at a dose level of 20,000 ppm of the tox-batch in the diet (Schoenmakers, 1994b).

Subchronic 90-day oral toxicity in rats: experimental design. In a subchronic 90-day toxicity study, the tox-batch was administered to SPF-bred Wistar rats in the diet. The study consisted of four groups, each comprising 10 males and 10 females. The dose levels in the diet were 0 (control), 2000, 10,000 and 50,000 ppm.

All animals were subjected to daily clinical observation. Body weight and food consumption were measured weekly and on the day before autopsy. Before the start of treatment and during wk 13 of treatment, both eyes of all animals of the control group and the highest treatment group (50,000 ppm) were examined by means of an ophthalmoscope. During wk 13, blood was collected from each animal for clinical laboratory investigations (Table 2). At the end of wk 13, all animals were autopsied and macroscopic observations and organ weights (adrenal glands, brain, heart, kidneys, liver, spleen and testis) recorded. The following organs were collected from all animals: adrenals, adrenal glands, aorta, brain, caecum, cervix, colon, duodenum, epididymides, eyes with optic nerve and Harderian gland, female mammary gland area, femur (including knee joint), heart, ileum, jejunum, kidneys, liver, lung (infused with formalin), lymph nodes (mandibular and mesenteric), oesophagus, ovaries, pancreas, pituitary gland, prostate gland, rectum, salivary glands (mandibular and sublingual), sciatic nerve, seminal vesicles, skeletal muscle, skin, spinal cord (cervical, midthoracic and lumbar), spleen, sternum (with bone marrow), stomach, testes, thymus, thyroid (including parathyroid), trachea, urinary bladder, uterus, vagina, and all gross lesions. All tissues collected from all animals of the control and highest dose group, as well as all gross lesions and lungs of all animals (all dose groups) were processed and slides were examined by a board-certified pathologist.

Subchronic 90-day oral toxicity in rats: results. Accuracy, stability and homogeneity of the tox-batch in the rat diet were demonstrated by analyses of the β-glucanase enzyme activity (Lecq, 1994a). There were no changes in body weights, food consumption, ophthalmoscopic findings, clinical biochemistry parameters, macroscopic findings, organ weights and microscopic findings that were considered to be an effect of treatment. In one female of the 50,000 ppm

Table 2. Laboratory investigation carried out in target species safety study in broiler chicks and subchronic toxicology study in rats

Parameter/Abbreviation	Unit	Broiler	Rat
HAEMATOLOGY (blood containing EDTA anti-coagulant)			
Erythrocyte count/RBC	T/litre	×	×
Haemoglobin/HB	mmol/litre	×	×
Haematocrit/HCT	l/litre	×	×
Mean corpuscular volume/MCV	fl	×	×
Mean corpuscular haemoglobin/MCH	fmol	×	×
Mean corpuscular haemoglobin concentration/MCHC	mmol/litre	×	×
Platelet count	g/litre	×	×
Red cell distribution width/RDW	%	×	×
Total leucocyte count/WBC	g/litre	×	×
Differential leucocyte count/SEG (Neutrophils), EO (Eosinophils), BASO (Basophils), LYMPH (Lymphocytes), MONO (Monocytes)	1 (rel)	×	×
Prothrombin time/PT*	sec		×
Partial thromboplastic time/PTT*	sec		×
CLINICAL BIOCHEMISTRY (serum samples)			
Alanine aminotransferase/(ALAT/GTP)	μkat/litre	×	×
Aspartate aminotransferase/(ASAT/GOT)	μkat/litre	×	×
Bilirubin, total/BILI T.	μmol/litre	×	×
Creatinine	μmol/litre		×
Creatine kinase	μkat/litre	×	
Glucose	mmol/litre	×	×
Lactase dehydrogenase	μkat/litre	×	
Urea	mmol/litre	×	
Protein, total/PROTEIN T.	g/litre	×	×
Protein, albumin/ALBUMIN	g/litre	×	×
Alkaline phosphatase/ALP	μkat/litre	×	×
Sodium	mmol/litre	×	×
Potassium	mmol/litre	×	×
Chloride	mmol/litre		×
Calcium	mmol/litre	×	×
Phosphorus/INORG. PHOSPH	mmol/litre		×

*Blood containing citrate as anticoagulant.

dose group, hypersensitivity to touch was observed at various times throughout the study. In addition, yellow staining of the fur was seen in females receiving 50,000 ppm in the diet. In females receiving 50,000 ppm of the tox-batch in the diet for 90 days, a slight hyperchromatic anaemia was noted. Red blood cell numbers, haematocrit and mean corpuscular haemoglobin concentration were altered, but no changes were seen in red cell volume or red cell distribution width (Table 4). Histopathological examination did not reveal any changes in spleen or liver, organs associated with blood cell formation or sequestration. With the information available it was not possible to elucidate a mechanism by which the anaemic condition could have occurred. Other haematological changes achieving statistical significance, in the female treated groups (10,000 and 50,000 ppm), were considered to be toxicologically non-significant, as they were too small and/or showed no dose relationship. No changes were seen in haematological values in females of the 2000 ppm group or treated males of all dose groups.

It was concluded that the feeding of the tox-batch at dietary levels up to 10,000 ppm did not induce noticeable signs of toxicity. The no-observed-effect level (NOEL) of the tox-batch in the subchronic study was therefore 10,000 ppm in the diet (Schoenmakers, 1995).

Irritation studies

Primary skin irritation/corrosion study in the rabbit. A 0.5-g aliquot of the tox-batch was applied to shaved skin of three male albino rabbits using semi-occlusive dressings for 4 hr, followed by four observations at approximately 1, 24, 48 and 72 hr after removal of the dressings and remaining test substance.

Table 3. Target species safety study in broiler chicks fed Barlican for 28 days: weight gain (WG), food conversion efficiency (FGR) and organ/body weight ratio

Dose			WG (g)		FGR†				Organ/BW ratio (%)‡			
Group*	ppm	BGU/kg	14 days	28 days	14 days	%	28 days	%	Heart	Liver	Kidneys	Spleen
1. (n = 90)	—	—	552	1397	1.651	100	1.889	100	0.6 ± 0.1	3.0 ± 0.3	0.65 ± 0.10	0.11 ± 0.03
2. (n = 90)	100	800	579	1416	1.568	95.0	1.829	96.8	—	—	—	—
3. (n = 90)	400	3200	586	1420	1.536	93.0	1.804	95.5	—	—	—	—
4. (n = 90)	800	6400	579	1420	1.514	91.7	1.774	93.9	0.6 ± 0.2	3.1 ± 0.4	0.58 ± 0.07	0.11 ± 0.03

— = not determined

*Organ/BW ratio of n = 24 chicks for control group and n = 12 for treated groups only.
†FGR = feed:gain ratio (feed conversion efficiency).
‡Values are means ± standard deviation.

Table 4. Subchronic toxicity study of Barlican tox-batch in rats; results of haematological investigations

	Treatment (ppm tox-batch)							
	Group 1 (control)		Group 2 (2000 ppm)		Group 3 (10000 ppm)		Group 4 (50000 ppm)	
Blood parameters‡	M	F	M	F	M	F	M	F
RBC	8.54	7.35	8.41	7.36	8.59	7.15	8.47	6.88**
(T/l)	0.39	0.29	0.34	0.14	0.36	0.30	0.29	0.27
HB	9.8	9.2	9.6	9.1	9.6	9.0	9.6	9.0
(mmol/litre)	0.4	0.2	0.3	0.3	0.3	0.3	0.4	0.3
HCT	0.442	0.404	0.434	0.401	0.438	0.389*	0.433	0.381**
(l/l)	0.020	0.012	0.012	0.012	0.016	0.014	0.018	0.012
MCV	51.8	55.0	51.7	54.5	51.0	54.4	51.1	55.4
(fl)	1.6	1.4	1.7	1.6	1.2	1.7	1.0	1.9
MCH	1.2	1.3	1.1	1.2	1.1	1.3	1.1	1.3**
(f/mol)	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
MCHC	22.3	22.9	22.0	22.8	22.0*	23.2	22.3	23.8**
(mmol/litre)	0.3	0.5	0.2	0.5	0.3	0.3	0.2	0.5
RDW	14.6	12.5	14.2	12.5	14.5	12.5	14.3	12.6
(%)	0.6	0.6	0.7	0.6	0.7	0.5	0.8	0.4
WBC	7.1	3.7	5.5*	3.4	5.6*	3.7	6.2	3.9
(g/litre)	1.6	0.9	1.0	0.5	1.5	1.0	1.2	1.2
SEG.	0.132	0.111	0.137	0.152	0.141	0.115	0.139	0.122
1	0.029	0.053	0.043	0.039	0.032	0.046	0.031	0.045
EO.	0.010	0.013	0.010	0.015	0.010	0.010	0.009	0.012
1	0.007	0.010	0.008	0.007	0.010	0.007	0.008	0.007
BASO.	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
1	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
MONO.	0.042	0.026	0.063†	0.036	0.034	0.030	0.034	0.036
1	0.019	0.013	0.011	0.018	0.017	0.012	0.021	0.019
LYMPH.	0.816	0.851	0.791	0.798	0.815	0.847	0.819	0.831
1	0.045	0.058	0.037	0.045	0.038	0.054	0.044	0.052
PLATELETS	879	808	813	783	787	810	830	720
(g/litre)	173	100	91	80	173	99	65	91
PT	15.2	15.5	14.7	15.1	14.4*	14.6**	14.7	14.8*
(sec)	0.8	0.5	0.6	0.4	1.0	0.8	0.5	0.6
PTT	17.7	17.0	17.5	17.7	18.7	17.5	18.2	16.4
(sec)	1.0	3.1	1.0	1.5	2.1	1.9	1.0	1.0

‡Abbreviations and units as in Table 2.
Asterisks and dagger indicate significant differences from corresponding controls (*$P < 0.05$; **$P < 0.01$; Dunnett's test based on pooled variance, or †$P < 0.05$; Steel's test).

The tox-batch resulted in very slight erythema in one animal, which resolved within 24 hr after exposure. No corrosive effects on the skin, no signs of systemic intoxication and no deaths were observed in any of the three rabbits during the study period. The tox-batch resulted in a primary irritation index of 0 (non-irritating) when applied to the intact rabbit skin. According to the EEC criteria for classification and labelling requirements (EEC, 1992a), the tox-batch does not require labelling as a skin irritant (Pels Rijcken, 1994a).

Acute eye irritation/corrosion study in the rabbit. A 30-mg aliquot of the tox-batch was instilled into one eye of each of three male albino rabbits, followed by four observations at approximately 1, 24, 48 and 72 hr after test substance administration.

Under the conditions of this study, the tox-batch resulted in adverse effects on the conjunctivae in all animals, which had resolved within 24 hr after instillation. No ocular corrosion or staining of peri-ocular tissues by the test substance was observed. Furthermore, no signs of systemic intoxication were observed during the study period and no deaths occurred. The tox-batch was considered practically non-irritating to the rabbit eye (Kay and Calandra interpretation of the calculated maximum Draize score: 2; Kay and Calandra, 1962). According to the EEC criteria for classification and labelling requirements, the tox-batch does not require labelling as an eye irritant (Pels Rijcken, 1994b).

Skin sensitization

Contact hypersensitivity in the albino guinea pig (maximization test). After identification of the slightly irritating and the highest non-irritating concentrations of the tox-batch in the preliminary study, a main study was performed with the selected concentrations. In the main study, 10 experimental animals were intradermally injected with a 5% concentration and epidermally exposed to 50% of the tox-batch, while at least five control animals were similarly treated but with the vehicle water only. Immediately after the last exposure, the skin irritation was scored. 2 wk after the epidermal application (=last induction) all animals were challenged with tox-batch concentrations of 50, 25 and 10% and the vehicle. The challenge reactions were assessed 24 and 48 hr after bandage removal.

Epidermal exposure of the tox-batch in the induction phase resulted in slight erythema in one experimental animal and a few very small crusts in two other animals (Table 5). The epidermal exposure of the tox-batch in the challenge phase, however, resulted in positive reactions in response to all three

concentrations of the tox-batch 24 and 48 hr after the challenge exposure. These reactions were characterized by erythema and/or swelling. Eschar formation and/or scaliness were also noted among the animals at several skin sites. In addition, no deaths occurred and no symptoms of systemic toxicity were observed in the animals during the study period.

It was concluded that the tox-batch resulted in a sensitization rate of 100%; was a strong sensitizer (Magnusson and Kligman, 1969). According to the EEC criteria (91/325/EEC) the tox-batch should be labelled as a skin sensitizer (Daamen, 1994b).

Inhalation study

Acute inhalation toxicity study in the rat. The acute inhalation toxicity of the tox-batch was studied by nose-only exposure of one group of five male and five female rats to a test atmosphere containing the maximally attainable concentration of 3.83 g/m^3 of the tox-batch for a single 4-hr period. The percentages of particles of diameter 4.2 μm or less was about 80%. After exposure, the rats were kept for a 15-day observation period. Body weight was measured weekly and just before autopsy. At the end of the observation period, all rats were killed, autopsied and examined for gross pathological changes.

A slight visually decreased breathing frequency was seen during the last hour of exposure, whereas no abnormalities were seen shortly after exposure or during the 15-day observation period. Normal body weight gain was generally seen during the observation period. A single or a few petechiae or white bump spots were seen on a lung lobe of three rats. In the other rats no abnormalities were seen. It was concluded that the 4-hr LC_{50} was more than 3.83 g/m^3 (Arts, 1994).

Mutagenic evaluation

Bacterial gene mutation test (Ames test, 'Salmonella typhimurium Reverse Mutation Assay'). The tox-batch was tested in the Ames test up to and including a concentration of 5000 μg/plate in both the absence and presence of a metabolic activation system (S-9 mix). The tox-batch did not induce a dose-related increase in the number of revertant (His$^+$) colonies in each of the four tester strains (TA1535, TA1537, TA98, TA100) in both the absence or the presence of S-9 mix. These results were confirmed in an independent repeat experiment. The positive controls gave the expected strong increase in the number of revertants (His$^+$) colonies in both the absence and presence of the S-9 mix. The tox-batch can, therefore, be considered as not mutagenic under the conditions of this test (Verhagen, 1994).

In vitro mammalian cytogenetic test: chromosome aberration test (CHO cells). The tox-batch was examined for its potential to induce structural chromosome aberrations in Chinese hamster ovary (CHO) cells, in both the absence and presence of S-9 mix up to and including a concentration of 5000 μg/ml.

In the first chromosome aberration assay, in the absence of the S-9 mix, the cells were exposed for 18 hr (approx. 1.5 times the cell cycle time) to the tox-batch; in the presence of the S-9 mix, the cells were exposed for 3 hr to the tox-batch, washed and cultured for an additional 15 hr. In both the absence and presence of the S-9 mix, three concentrations of the tox-batch (1000, 3000 and 5000 μg/ml), the negative (vehicle) and positive control were selected for chromosomal aberration analysis. In the second (independent) chromosome aberration assay in the absence of the S-9 mix, the cells were exposed for 18 and 32 hr to the tox-batch; in the presence of the S-9 mix, the cells were exposed for 3 hr to the tox-batch, washed and cultured for an additional 15 or 29 hr. At the harvesting time of 18 hr in both the absence and the presence of the S-9 mix, three concentrations of the tox-batch (1250, 2500 and 5000 μg/ml) together with the negative (vehicle) and positive control, were selected for the analysis of chromosomal aberrations; at the harvesting time of 32 hr (approx. 2.5 times the cell cycle time), in both the absence and presence of the S-9 mix, one concentration of the tox-batch (5000 μg/ml) together with the negative (vehicle) control, were selected for the analysis of chromosomal aberrations.

In both the first and the second chromosome aberration assay, the tox-batch did not induce a statistically significant increase in the percentage of cells with structural chromosome aberrations at any of the concentrations and time points analysed, when

Table 5. Hypersensitivity of albino guinea pigs to Barlican tox-batch (maximization test): induction and challenge readings

	Induction Day 10 readings*		Challenge Day 24 readings				Challenge Day 25 readings			
	50%†									
Group	Erythema	Oedema	50%†	25%	10%	0%	50%†	25%	10%	0%
Experimental group (n = 10)	1/10[c]	0/10	10/10[c]	10/10	10/10	0/10	10/10[cs]	10/10[s]	10/10[cs]	0
Control group (n = 5)	0/5	0/5	0/5	0/5	0/5	0/5	1/5	0/5	0/5	0/5

*Erythema and oedema scores 1–4.
†Percentages indicate concentration in distilled water.
[s]Scaliness in some animals.
[c]Eschar formation in some animals.
[c]Few very small crusts in some animals.

compared with the vehicle control values. The positive control substances, mitomycin C (in the absence of the S-9 mix) and cyclophosphamide (in the presence of the S-9 mix), induced the expected increases in the incidence of structural chromosome aberrations in both chromosome aberration assays.

In conclusion, the tox-batch is considered to be not clastogenic, under the conditions of this test (de Vogel, 1994).

DISCUSSION

A number of safety studies were performed to investigate the safety for use of the tox-batch of β-glucanase enzyme from *Trichoderma reesei*. A strong skin-sensitizing potential of the tox-batch detected in the sensitive guinea pig model indicates that there is a potential risk for the workers in the poultry feed industry when handling the enzyme preparation. The sensitizing potential to humans can be judged from test results in guinea pigs only by taking into account the probable human exposure (Botham *et al.*, 1991). Since exposure may be influenced by several variables, such as the nature of formulation of the enzyme product and the frequency and duration of exposure, the following manufacturing procedures should be noted.

During the regular production of the enzyme powder in the Gist-brocades plants, sufficient precautions are taken to avoid inhalation and skin contact. In addition, the formulation of the enzyme preparation mainly takes place in a tightly closed mixing apparatus, except during the standardization of enzyme powder and wheat flour and during regular cleaning procedures. At these stages the workers should take precautions to avoid inhalation and skin contact. Finally, in the feed compounding industries Barlican is added to barley-based poultry diets in concentrations of approximately 100 ppm (0.01%, w/w). According to the EEC directive on dangerous preparations and substances, preparations containing less than 1% of a sensitizer should not be labelled (Botham *et al.*, 1991). The risks of sensitization are considered to be acceptable when taking into consideration (a) the strong sensitizing potential of the tox-batch by epidermal exposure, (b) the normal occupational health precautions taken in the manufacture and use of enzymes, (c) the extremely low exposure of workers in the feed-compounding industry who take due precautions and (d) the fact that strong sensitizers may be used safely if there is a short period of contact between a low concentration and a normal skin (Botham *et al.*, 1991).

No evidence of oral or inhalation toxicity, mutagenic potential, eye or skin irritancy was found. Furthermore, the NOEL of the subchronic oral toxicity study in rats can be used in calculation of the safety margins based on the concentration of the enzyme in feed products and on data of the target animal feed consumption. Feeding the tox-batch at dietary levels of 10,000 ppm in the subchronic toxicity study in rats did not induce noticeable signs of toxicity. The NOEL of the tox-batch in the rat study was therefore 10,000 ppm, corresponding in the 90-day study to an overall intake of 824–1028 mg/kg body weight/day (22,083–27,550 BGU/kg body weight/day) for male and female rats, respectively (Schoenmakers, 1995).

During the 28-day target species safety study in broiler chicks, no evidence of toxicity was observed at dose levels of 800 ppm (6400 BGU/kg) of Barlican (i.e. eight times the recommended dose, 800 BGU/kg, in broiler feed) in the diet. In addition, the mean daily feed consumption for broiler chicks will not exceed 100 g/kg body weight, which is equivalent to an addition of 80 BGU/kg. Comparing this with the NOEL in rats of at least 24,000 BGU/kg/day, there is a safety factor of 300 or more.

According to the literature, the production organism *T. reesei* is non-pathogenic and non-toxicogenic. Toxins have not been detected in the investigations undertaken on the tox-batch or the commercial enzyme preparation. The production organism is not present in the final (commercial) preparations.

In conclusion, the results of the efficacy and safety studies of the β-glucanase enzyme preparation in this review confirm the safety of Barlican in the intended target species under the recommended dose levels and given during the normal period of feeding, in common practice. However, some additional occupational health precautions should be taken during the manufacturing and use of the enzyme preparation, with regard to the sensitizing potential.

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