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Safety Evaluation of a Lipase Expressed in *Aspergillus oryzae*

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Abstract—A programme of studies was conducted to establish the safety of a lipase artificially expressed in *Aspergillus oryzae* to be used in the detergent industry and as a processing aid in the baking industry. Laboratory animal studies were used to assess general and inhalation toxicity, skin sensitization, and skin and eye irritation. Its potential to cause mutagenicity and chromosomal aberrations was assessed in microbial and tissue culture *in vitro* studies. The pathogenicity of *A. oryzae*, the organism used to produce the lipase, was also assessed in laboratory animals. Basic ecotoxicity in a variety of test species was studied. General and inhalation toxicity was low. There was evidence of mild skin irritation. There was no evidence of eye irritation, skin sensitization, mutagenic potential, chromosomal aberrations, ecotoxicity or notable pathogenicity. Comparison of these results with human exposure levels and previously published data indicates that the lipase appears safe for consumers in the given applications, requires no special occupational health precautions in manufacture and is of low environmental impact. Furthermore, the organism used in production of the lipase has no notable pathogenicity.

INTRODUCTION

Lipases are triglyceride-hydrolysing enzymes which occur widely in animals, plants and micro-organisms. Lipases have different properties depending on fatty acid specificity, positional specificity, thermostability, pH optimum, etc., and a number of industrial applications have emerged (Eigtved, 1992).

Early use of lipases has been in flavour enhancement of cheese by release of specific short-chain fatty acids.

In the detergent industry lipases are used to decompose fatty stains into more hydrophilic substances (Aaslyng *et al.*, 1991). Lipase-catalysed interesterification of fats and synthesis of fatty acid esters are used in the production of emulsifiers and speciality fats.

The application of lipases in the baking industry is rather new (Qi Si and Hansen, 1994). The lipase can be added in a granulated form, either to the flour or dough in a dose range of 0.05 to 0.5 ppm enzyme protein. This enzyme treatment results in improved dough properties and bread-making quality in terms of larger volume, whiter crumb colour, improved shelf-life and crumb structure. The observed effects can be explained by a mechanism in which the lipase changes the interaction between gluten and some lipid fragments during dough mixing.

Abbreviations: CPA = cyclophosphamide; DOC = dissolved organic carbon; EC_{50} = median effective concentration; EDI = estimated daily intake; LC_{50} = median lethal concentration; LD = lethal dose; LD_{50} = median lethal dose; LU = lipase unit; NOEL = no-observed-effect level; PII = primary irritation index; TOS = total organic substance.

MATERIALS AND METHODS

The test batches of *Humicola lanuginosa* lipase artificially expressed in *Aspergillus oryzae* used for all safety studies were produced in the same manner as in production scale.

The batch designated test material 'A' was used in the following studies: skin irritation, acute inhalation toxicity, subacute toxicity, subchronic toxicity, two mutagenicity studies, two tests in aquatic organisms and biodegradability. The batch designated test material 'B' was used in the acute oral toxicity test. The batch designated test material 'C' was used for skin irritation, eye irritation and delayed contact sensitization studies. The batch designated 'D' was used in the algal growth inhibition test.

In the references cited the lipase appears under the names: SP 400 and lipolase.

Spores of the two strains of *A. oryzae*, namely H-1-52 and A1560, were used to investigate relative pathogenicity.

Key characteristics among the test material specifications are given in Table 1.

Toxicity studies

Acute oral toxicity in rats (Stavnsbjerg, 1988a). Test batch B was suspended in tap water and given once orally by gavage to groups of five male and five

female animals after overnight fasting. Wistar rats weighing 70–76 g were given 5 g/kg. The animals were observed daily for 14 days after dosing and were then killed and subjected to autopsy.

The rats showed no clinical signs, and all survived treatment and the 14-day observation period. The median lethal dose (LD_{50}) was therefore demonstrated to exceed 5 g/kg.

Acute inhalation toxicity in rats (McDonald, 1988). A group of five male and five female Sprague–Dawley rats weighing 115–142 g were confined in a nose-only inhalation chamber and exposed for 4 hr to an atmospheric concentration of 0.74 ± 0.1 mg/litre, this being the highest concentration that could be maintained over the 4-hr period. Test batch A was used as supplied and the test atmosphere was generated by means of an Aerostyle (Aerosyte Co. Ltd, London, UK) dust generator. Particle size distribution studies indicated that the mass mean diameter was 8.2 μm with a geometric standard deviation of 3.0 μm.

The inhalation median lethal concentration (LC_{50}) was not demonstrated in this limit test other than an indication that the value exceeded 0.74 mg/litre, this being the highest concentration that could be maintained over the exposure period.

Subacute oral toxicity studies in rats. In a 2-wk study (McDonald and Parkinson, 1988) groups of 10 male and 10 female Sprague–Dawley rats were dosed once per day by gavage at dose levels of 0 (control), 0.2, 2 or 10 g/kg. Test batch A was suspended in distilled water vehicle and administered at a dose volume of 10 ml/kg.

In a subsequent 13-wk study (Perry *et al.*, 1989) groups of 20 male and 20 female Sprague–Dawley rats were administered test batch A dissolved in distilled water at a dose volume of 10 ml/kg to give dose levels of 0, 0.2, 1 and 5 g/kg.

Routine clinical observations, body weights and food consumption measurements were undertaken throughout the study periods. Terminal haematology and clinical chemistry investigations were performed and on completion of the respective treatment periods all animals were subjected to gross pathological examination and organ weight analyses. Microscopic examination of a comprehensive list of tissues was undertaken for control and high-dose animals in both studies.

No treatment-related clinical signs were seen in either study. Body weight gain, and food and water consumption were not affected by treatment. Haematology and clinical chemistry investigations did not reveal any changes. Gross and microscopic pathology and organ weight analysis gave no indication of adverse responses to treatment.

Table 1. Characteristics of the lipase test batches

	Test batch			
	A	B	C	D
Enzyme activity* (10^6 LU/g)	0.208	0.127	0.0823	0.925
N_{total} × 6.25 (%, w/w)	17.0	14.9	6.13	36.5
$N_{protein}$ × 6.25 (%, w/w)	7.38	6.94	4.4	28.7
Water (%)	67.0	68.5	85.1	5.2
TOS† (%)	27.0	25.2	11.8	43.8

LU = lipase unit TOS = total organic substance

One LU is defined as the amount of enzyme which liberates 1 μmol of titratable butyric acid under standard conditions [4.8% (w/v) tributyrin emulsion with 0.1% (w/v) gum arabic, pH 7.0, 30°C].

*The specific enzyme activity is 5.0×10^5 LU/g and defined as the activity (LU) of 1 g of pure enzyme protein.

†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 (no diluents were added).

Irritation studies

Skin irritation in rabbits (Berg, 1988b,d and 1990). Three primary skin irritation tests were carried out (two with test batch C and one with test batch A mixed with equal volumes of propyleneglycol) following the method of the Code of Federal Regulations (1979), adapted to OECD Guidelines for Testing of Chemicals (1981a). The backs of 12 albino rabbits were clipped free of hair and 0.5 g of test material was introduced under gauze (2.5 × 2.5 cm). Test sites for two of the tests were divided into abraded and intact skin. Patches were secured for the 4-hr exposure period and covered with impervious material. Patches and the remaining test material were removed with water after 4 hr and any skin reactions were evaluated at approximately 1, 24, 48 and 72 hr after removal. A primary irritation index (PII) was calculated and used to classify the test material: 0, non-irritant; $0 \leqslant 2$, mild irritant; $>2 < 5$, moderate irritant; and >5, severe irritant.

Test batch C had a PII of 0 and 0.27 and was thus classified as a mild irritant to skin; test batch A had a PII of 0 and was classified as non-irritant to skin. The mild irritant category for batch C resulted from four rabbits having very slight erythema and oedema at the dosing site 0.5–72 hr after patch removal.

Eye irritation in rabbits (Berg, 1988a). An eye irritation test was carried out with test batch C following the OECD (1981b) with evaluations made following the Code of Federal Regulations (1980). Three albino rabbits were used, with the equivalent of approximately 0.1 ml of test material being instilled into the conjuctival sac of the left eye and the grade of ocular reaction being recorded at 1, 24, 48 and 72 hr after instillation. The test material produced no corneal or iris reactions at any time point but produced conjuctival reddening in one rabbit after 1 hr only but not after an extended observation period of 7 days. As a result, this batch was regarded as negative in its potential for eye injury.

Skin sensitization: delayed contact hypersensitivity in guinea pigs (Berg, 1988c). A modified Draize test was performed after the method described by Johnson and Goodwin (1985). Test batch C was tested for induction and challenge procedures on groups of 20 guinea pigs each.

The test consisted of an induction phase, followed by a resting period and a subsequent challenge phase to prove whether sensitization occurred. The induction was performed by four intradermal injections of 0.1 ml of test material at a concentration of 500 LU/g, at sites overlying the axillary and inguinal lymph nodes in 20 animals. 10 animals were injected similarly with 0.9% sterile saline (negative control). All animals were challenged epicutaneously using a 6-hr occluded patch 13 days later with test material (30,000 LU/g). 1 wk later all animals were challenged intradermally in the flank with 0.1 ml test material (200 LU/g). All animals were rechallenged epicutaneously 1 wk later to confirm the first epicutaneous challenge.

At the first epicutaneous challenge positive responses were seen in three of 20 test animals, whereas negative responses were noted in all the control animals. The intradermal challenge did not reveal any significant difference between test and control animals and the second epicutaneous challenge produced positive responses in one test animal and four control animals. Negative results were recorded in both the rechallenge and irritancy groups. It was therefore concluded that sensitization had not been shown with the lipase.

Mutagenicity

Gene mutation (Pedersen, 1988). Test batch A was examined for mutagenic activity using *Salmonella typhimurium* strains TA1535, TA100, TA1537 and TA98 and *Escherichia coli* WP2uvrA (pKM101).

Lipase activity was removed from the test material by ultrafiltration before testing because of its destructive action on the metabolic activation system (S-9) and the bacterial cell wall.

A liquid culture assay was used. Bacteria were exposed to five doses of test material (from 0.1 to 10 mg/ml incubation mixture at half-log intervals) in a phosphate buffered nutrient broth for 3 hr. After incubation the test substance was removed by centrifugation before plating. The numbers of revertants to prototrophy and viable cells were estimated.

The test was conducted in the presence and absence of metabolic activation, namely, a liver preparation from male rats pretreated with Aroclor 1254 and cofactors required for mixed function oxidase activity (S-9 mix).

The sensitivity of the individual bacterial strains was confirmed by notable increases in the number of revertant colonies induced in similar liquid conditions by diagnostic mutagens.

No dose-related increases in revertants to phototrophy were obtained in any of the tests performed; all results were confirmed in an independent experiment and it was concluded that there were no indications of mutagenic activity in the presence or absence of metabolic activation.

Chromosome aberrations (Marshall, 1988). Test batch A was tested in an *in vitro* cytogenetics assay using human lymphocyte cultures from a male and a female donor. Treatments were performed in the absence and presence of metabolic activation by a rat liver post-mitochondrial fraction (S-9) from Aroclor-1254 induced animals. The test compound dose levels for analysis were selected by determining mitotic indices from a broad range of doses up to 5000 μg/ml. There was no clear evidence of any treatment-related mitotic inhibition at any of the dose levels analysed.

Appropriate negative (solvent) control cultures were included in the test system and contained low incidences of chromosomal aberrations within historical solvent control ranges. Methyl methanesulfonate and cyclophosphamide (CPA) were used as positive control chemicals in the absence and presence of liver S-9, respectively. Both compounds induced statistically significant increases in the incidence of chromosomal aberrations. It was considered important that the presence of lipase did not affect the activity of the S-9 liver preparation. This was achieved by showing that S-9-mediated clastogenicity of CPA was not reduced in the presence of test material up to 5000 μg/ml.

Treatment of cells with test material in both the absence and presence of S-9 resulted in numbers of aberrations which were similar to those observed in concurrent negative controls. A small but statistically significant increase in "total aberrations including gaps" which pushed the category total in the female donor outside the normal range, was observed at the intermediate dose level in the absence of S-9 but was not considered to be of biological significance. There were no significant differences between treated cells and controls at any other dose level in either the absence or presence of S-9 and aberration frequencies fell within historical control ranges.

It is concluded that the test material was unable to induce chromosome aberrations in human lymphocytes when tested up to 5000 μg/ml in either the absence or presence of S-9.

Ecotoxicology

Aquatic organism toxicity (Bogers, 1989a,b; Douglas and King, 1993). Concentrations of test batch A were tested for their ability to immobilize the water flea *Daphnia magna* over a 24-hr period under static conditions (OECD, 1981a). Five *Daphnia* were placed in a test-tube containing an aqueous solution of test material at 1 g/litre. Three such test-tubes were used at each concentration. An equivalent study was simultaneously conducted using concentrations of aqueous potassium dichromate to provide a positive control. No immobilization was seen after 24 hr of exposure.

The actual concentration of the test substance expressed as amount of enzyme activity was examined by analysis of duplicate samples taken from

1 g/litre and the blank during the main study at 0, 2 and 24 hr.

Analysis of samples taken from the limit concentration revealed a significant loss of enzyme activity during the 24 hr of exposure. The mean value for enzyme activity was 82% after 2 hr and 76% after 24 hr of exposure compared with that measured at 0 hr. No enzyme activity was detected in samples taken from the blank control.

The 24-hr median effective concentration (EC_{50}) for immobility in *Daphnia* exposed to test material was more than 1 g/litre.

A 96-hr semistatic test for acute toxicity in the carp (OECD, 1981e) was also carried out. 10 carp (*Cyprimus carpio*) were maintained in an aquarium containing an aqueous solution of test batch A at 1 g/litre. Solutions were renewed every 48 hr. An equivalent study was carried out using concentrations of pentachlorophenol to provide a positive control. No mortality or other effects were observed after 96 hr of exposure.

The actual concentration of the test substance expressed as amount of enzyme activity was examined by analysis of duplicate samples taken from 1 g/litre and the blank during the main study at 0, 2, 24 and 48 hr.

Analysis of samples taken from the limit concentration revealed no significant loss of enzyme activity during the first 48 hr of exposure before renewal of the test media. Enzyme activity was 96% after 2 hr of exposure, 91% after 24 hr and 95% after 48 hr compared with that measured at 0 hr. No enzyme activity was detected in samples taken from the blank control. The 96-hr LC_{50} appeared to be more than 1 g/litre.

Algal growth inhibition test. An algal growth inhibition study was performed (in accordance with OECD, 1981c) to assess the inhibitory effect of test material on the growth of the unicellular green alga *Scenedesmus subspicatus* strain no. CCAP 276/20.

Algal cultures were exposed to five concentrations (10–160 mg/litre of test batch D, each in triplicate) of test material plus one untreated control. These were incubated on an orbital shaker under continuous illumination at 24 ± 1°C for 72 hr. Growth was monitored daily by measuring the absorbance of each culture at 665 nm.

The EC_{50} for inhibition of growth after 72 hr was 97 mg/litre and the EC_{50} for inhibition of maximum growth rates (24–72 hr) was 99 mg/litre. The no-observed-effect level (NOEL) was 40 mg/litre. All results were based on nominal concentrations.

Biodegradability (Bogers, 1989c; Douglas and James, 1991)

A biodegradability test with recording of dissolved organic carbon (DOC) was performed (OECD, 1981f). The inoculum used was freshly collected activated sludge from domestic sewage. Sealed flasks were maintained in the dark at 22°C for 28 days with continuous stirring. Flasks were organized as: (i) inoculated medium with 250 mg test material (duplicate flasks); (ii) inoculated medium only (duplicate flasks); and (iii) inoculated medium with 90 mg sodium benzoate/litre (duplicate flasks) (inoculum activity test with reference compound). The DOC in each flask was measured with respect to time.

Test batch A attained 99% biodegradation within 28 days. The pass level of 70% was reached within 10 days of exceeding the 10% level and consequently the test material can be termed as readily biodegradable. The standard substance, sodium benzoate, attained 95% biodegradation within 28 days, thereby confirming the suitability of the inoculum and the culture conditions.

Test batch A was also tested for its ready biodegradability in the closed bottle test at concentrations of 5 and 25 mg/litre. Oxygen demand was determined to be 0.435 mg/mg test material. At the low concentration of test material (5 mg/litre) the biodegradation reached 73% after 28 days of incubation. Biodegradation at the high concentration of test material (25 mg/litre) reached 78% after 28 days of incubation. However, biodegradation of at least 60% was not reached within 10 days of biodegradation exceeding 10%. Although substantial biodegradation was recorded after 28 days of incubation, test batch A appeared not to be readily biodegradable in the closed bottle test. Under the same conditions sodium acetate biodegradation reached 73%. Finally, test batch A appeared not to be toxic to the microbial activity present in the medium.

Pathogenicity of Aspergillus oryzae (Stavnsbjerg, 1988b)

The recombinant strain of the lipase-producing micro-organism *A. oryzae* was subjected to a pathogenicity study in mice comparing the pathogenic potential of the recombinant strain (H-1-52) with that of the host organism (A1560).

Groups of five NMRI mice were dosed intravenously with 1.4×10^9, 1.4×10^7 or 1.4×10^5 spores of strain A1560/kg body weight on one occasion. A further four identical groups of mice received 1.9×10^9, 1.9×10^7 or 1.9×10^5 spores of strain H-1-52/kg body weight. A further group received sterile sodium chloride solution and acted as controls. All mice received 10 ml/kg body weight. The mice were observed for 21 days for mortality and clinical symptoms. Autopsy was performed at termination, with liver, spleen, lung, kidney and brain examined histologically.

This investigation showed that spores of the fungus *A. oryzae* strains A1560 and H-1-52 can cause the death of mice when administered intravenously as a spore suspension of more than 10^5 cfu/kg body weight. The severity of the reactions observed was clearly dose dependent for both strains. The deaths were seen early in the observation period for the high-dose groups. The deaths were delayed up to 8

days for the intermediate groups, if the animals did not survive until termination.

The clinical symptoms of significance were symptoms arising from the central nervous system. Symptoms were observed only in animals dosed with strain A1560, but this cannot acquit strain H-1-52 of the ability to affect the central nervous system. The symptoms could have been missed. Histological examination of the brains available could not confirm the clinical symptoms. The gross pathological findings of significance were petechial haemorrhage in the lungs and enlarged kidneys. Subcutaneous haemorrhage in the parietal region of the skull was not regarded as an effect of treatment.

Histopathological examination revealed findings which combine the gross pathological findings with the expected injury from the artificial septicaemia. Heavy fungal growth was seen in the organs of animals in the high-dose groups, most commonly in the lungs. Pulmonary growth in animals dosed with strain H-1-52 caused tissue reaction in all five animals. For animals dosed with strain A1560 pulmonary growth with tissue reaction was seen in only two animals and one had renal growth with inflammatory cell infiltration. Fungal growth without tissue reaction in many organs seems to indicate that growth occurred after death. Hyperaemia in many organs without any other tissue reaction indicated passive congestion. The significant finding in the intermediate groups was renal mycotic growth with inflammation. No mycotic growth was detected in the lowest dose groups.

The lethal dose (LD_{50}) was approximately 10^6–10^7 spores/kg body weight in this study. The magnitude of the LD was within the same order of magnitude as published by Ford and Friedman (1967). The observed reactions are as expected when mice are exposed to high concentrations of *Aspergilli* spores by intravenous injection.

DISCUSSION AND CONCLUSION

A previous publication indicates that the safety evaluation of microbial enzymes should be based on knowledge of the source micro-organisms and toxicology testing of the enzyme preparation in animal models (Battershill, 1993). The adverse effect levels from such toxicology studies can then be used in the calculation of safety margins based on the enzyme's applications, concentration of the enzyme total organic substance (TOS) residues in final products and human consumption data. A theoretical calculation of human exposure to the lipase through consumption of bread can be made on the following assumptions:

The maximum dose is 2500 LU/kg flour, corresponding to 5 g of a 50 kLU/g lipase granulate per 100 kg flour or 0.5 ppm lipase protein.

The lipase granulate has a TOS content of 5%. Bread contains approximately 75% flour.

Maximum TOS in bread is then: 1.88×10^{-6} g/g bread.

Most, if not all, of the enzyme will be inactivated during the baking process.

An average bread consumption of 160 g/person/day and a body weight of 60 kg results in: 2.67 g bread/kg body weight/day.

The maximum estimated daily intake (EDI) of TOS through bread is therefore: 5.00 μg TOS/kg body weight/day.

The TOS content of test batch A was 27%.

The maximum NOEL used in the 13-wk rat study with test batch A was 5 g enzyme concentrate/kg/day or 5×0.27 g TOS/kg/day, giving: NOEL = 1.35 g TOS/kg/day.

The safety margin: NOEL/EDI = 2.70×10^5.

On this basis it can be concluded that the *H. lanuginosa* lipase artificially expressed in *A. oryzae* can be considered Generally Recognized As Safe. Published data show that *A. oryzae* is regarded as non-toxigenic and non-pathogenic. Inhalation, irritation and sensitization studies indicate that no special occupational health precautions need be applied in the manufacture and use of lipolase.

A. oryzae has been used in the Orient to produce koji, a complex enzyme preparation for the production of soy sauce, miso and sake for more than 2000 years. The micro-organism occurs naturally at some locations in China and Japan. Outside this area the fungus may be sporadically isolated from soil or decaying plant material (Domsch *et al.*, 1980). The fungus needs a relatively high growth temperature and this compares well with the few reports on the recovery of *A. oryzae* in temperature climates. *A. oryzae* belongs to the flavus group (Raper and Fennell, 1965); however, it differs from other species in the flavus group, but not for the fact that it does not produce aflatoxins.

Both *A. oryzae* and its enzymes are accepted as constituents of food (JEFCA, 1987). The safety of *A. oryzae* has been evaluated by a comprehensive literature survey in medical databases and by Barbesgaard *et al.* (1992). Case stories involving *A. oryzae* are extremely few and taking this into consideration the use of the fungus gives evidence for classifying the organism as non-pathogenic.

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