

Review Section

SAFETY EVALUATION OF ALKALINE CELLULASE

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Summary—A programme of studies was conducted to establish the safety of alkaline cellulase, an enzyme used as a processing aid in the food industry. Laboratory animal studies were used to assess general, inhalation and reproduction toxicity, eye irritation and skin irritation and sensitization. Mutagenic potential was assessed in microbial and animal *in vivo* studies. The pathogenicity of *Humicola insolens*, the micro-organism used in the production of alkaline cellulase, was also assessed in laboratory animals. Basic ecotoxicity in a variety of test species was studied. General toxicity by a variety of routes was low; there was no evidence of reproductive toxicity. There was evidence of mild skin irritation and some eye conjunctival reddening. There was no evidence of skin sensitization, mutagenic potential, ecotoxicity or notable pathogenicity. When these results are considered along with levels of human exposure and previously published data, it appears that alkaline cellulase is safe for consumers in the given applications, requires no special occupational health precautions in manufacture, and is of low environmental impact. Furthermore, the micro-organism used in production of alkaline cellulase has no notable pathogenicity.

Introduction

Cellulases have a long history of use in the food processing industry. Alkaline cellulase is made by submerged fermentation of a selected strain of the fungus *Humicola insolens*. It is a heat-stable multi-active enzyme preparation with β -glucanase, cellulase, xylanase, pentosanase and arabanase activity, and is used in brewing and baking.

This review summarizes a programme of studies that was conducted to establish the safety of alkaline cellulase when used as a processing aid in the food industry. The programme was designed to investigate possible effects on consumer and industrial occupational health and basic ecotoxicological impact. The enzyme product was investigated for general toxicity, effects on reproduction, mutagenic potential, inhalation toxicity, irritation to the skin and eye, skin sensitization, and ecotoxicity. The micro-organism *H. insolens* was also tested for its pathogenic potential. The studies were undertaken during the period 1982–1990.

Test material

Three batches of alkaline cellulase SP 227 were used in the safety evaluation programme. Batch no.

PPC 1247 was a sample of the commercial preparation and was used in early skin and eye irritation studies. Batches PPC 1317 (toxicity batch) and PPC 2809 were used in the remaining studies.

The characterization and chemical composition of batches PPC 1317 and PPC 2809 are shown in Table 1. The enzyme activity of these batches was 817 and 3200 CMCU/g, respectively, whilst that of the commercial product batch PPC 1247 was 969 CMCU/g. [The CMCU or carboxymethyl cellulose unit is the amount of enzyme that forms an amount of reducing carbohydrates equivalent to 1 μ mol glucose/unit under the standard conditions of 40°C, pH 7, carboxymethylcellulose in phosphate buffer.]

The analyses of antimicrobial activity undertaken on batches PPC 1317 and PPC 2809 revealed no antimicrobial agents. Total organic substance (TOS defined as 100%—(A + W + D)%), where A = ash content, W = water content and D = content of diluents) was 70.38% for batch PPC 1317 and 69.12% for batch PPC 2809. The stability of the SP 227 batches used in the safety evaluation programme was confirmed by determinations of enzyme activity.

Toxicity studies

Acute oral toxicity in rats and mice (Agger, 1982a,b)

The toxicity batch PPC 1317 was suspended in tap-water and was given as a single oral dose by gavage to groups of five male and five female animals after overnight fasting. Mice of the NMRI strain

Abbreviations: BOD = biochemical oxygen demand; CMCU = carboxymethylcellulose unit; DNCB = dinitrochlorobenzene; IC(I) = immobilization (initial) concentration; LC(I) = lethal (initial) concentration; PII = primary irritation index; TOC = total organic carbon.

Table 1. Microbial analysis and chemical composition of SP 227 Batches PPC 1317 and PPC 2809

Parameter	Batch no. PPC 1317	Batch no. PPC 2809
Microbial analysis		
Total viable counts/g	< 10	3×10^2
Spores/g	< 10	< 10^2
Enterobacteria/g	0	0
<i>Staphylococcus aureus</i> /g	0	0
Clostridia/g	< 10	< 10
<i>Bacillus cereus</i> /g	0	0
<i>Bacillus thuringiensis</i> /g	NA	< 10^1
<i>Pseudomonas aeruginosa</i> in 10 g	ND	ND
Fungi/g	< 10	< 10
Yeast/g	< 10	< 10
Streptococci/g	< 10^2	< 10^2
Salmonella in 50 g	ND	ND
Chemical composition		
N _{total} (%)	7.62	6.37
N _{protein} (%)	2.14	2.17
H ₂ O (%)	2.12	6.3
Carbohydrate, total (%)	41.2	29.6
Petroleum ether soluble residue (%)	< 0.1	NA
Na (%)	4.4	2.4
K (%)	9.6	6.3
Mg (%)	0.57	0.40
Ca (%)	0.87	4.0
NH ₄ (%)	2.43	1.93
Cl (%)	0.2	11.1
PO ₄ (%)	8.9	4.3
SO ₄ (%)	0.9	9.2
CO ₂ (%)	< 0.1	< 0.2
Heavy metals (ppm)	< 40	< 40
Pb (ppm)	< 8	< 10
As (ppm)	< 2	< 3
Ash (600 C) (%)	27.5	24.6

weighing 18–23 g were treated with 5, 7.5, 10 or 12.5 g/kg body weight and Wistar rats weighing 65–84 g were given 2.5, 5, 7.5 or 10 g/kg. The animals were observed daily for 14 days after dosing and were then killed and autopsied.

Dose-related decreased motor activity and diarrhoea was observed in the mice. The LD₅₀ in mice was calculated to be 8 g/kg body weight (95% confidence limits, 7.5–8.6). The rats showed no clinical signs and all of them survived treatment and the 14-day observation period; therefore the LD₅₀ was demonstrated to exceed 10 g/kg.

Acute intraperitoneal toxicity in mice (Agger, 1982c)

Groups of five male and five female NMRI mice weighing 19–23 g were dosed once ip with PPC 1317 dissolved in sterile physiological saline. Dose levels of 0.5, 1 and 2 g/kg body weight were used and resulted in a dose-related incidence of decreased motor activity, unsteady gait and tremor. No deaths were observed and the LD₅₀ was demonstrated to exceed 2 g/kg.

Alkaline cellulase can therefore be classified as 'practically non-toxic'.

Acute inhalation toxicity in rats (Greenough and McDonald, 1984)

A group of five male and five female Sprague–Dawley rats weighing 176–195 g were confined in a nose-only inhalation chamber and exposed for 4 hr to an atmospheric concentration of 3.48 ± 0.6 mg alkaline cellulase/litre, this being the highest concentration that could be maintained over the 4-hr period.

Batch PPC 1317 was used as supplied, and the test atmosphere was generated by means of a Wright dust feeder. Particle size distribution studies indicated that the mass mean diameter was $7.8 \mu\text{m}$ with a geometric standard deviation of 2.1 μm .

The inhalation LC₅₀ was not demonstrated in this limit test other than to indicate that the value exceeded 3.48 mg/litre.

Sub-acute oral toxicity studies in rats

In a 4-wk study (Husband and Wood, 1983), groups of five male and five female Sprague–Dawley rats were dosed once a day by gavage at dose levels of 0 (control), 0.3, 1 or 3 g/kg body weight. SP 227 (Batch PPC 1317) was suspended in distilled water and administered at a dose volume of 10 ml/kg. In a subsequent 13-wk study (Perry *et al.*, 1990) groups of 20 male and 20 female Sprague–Dawley rats were fed diets formulated with SP 227 (Batch no. PPC 2809) to give dose levels of 0, 0.12, 0.6 and 3 g/kg body weight.

Routine clinical observations, and determinations of body weights and food consumption were undertaken throughout the study periods. Haematological and clinical chemical investigations were carried out at the end of the treatment periods and all rats were subjected to gross pathological examination and organ-weight analyses. Microscopic examination was carried out on a comprehensive list of tissues taken from animals in the control and high-dose groups in both studies.

No treatment-related clinical signs were seen in either study. There was a slight dose-related increase in body-weight gain (approx. 7%) in the males from the 4-wk study and a reduction in body-weight gain in both sexes at the high-dose level in the 13-wk study (10% in males and 20% in females). A slight reduction in food consumption was also noted for the high-dose rats in the 13-wk study. Water consumption was not affected by treatment in either study.

Haematological and clinical chemical investigations did not reveal any changes after 4 wk of treatment. In the 13-wk study, there was an increase in alkaline phosphatase of 26% in intermediate-dose females and 68% in high-dose females. Gross and microscopic pathology and organ-weight analysis gave no indication of adverse responses to treatment in either study.

Teratogenicity in rats (McCay and Barton, 1990)

Following assessment of existing toxicity data, groups of 25 Sprague–Dawley rats were given SP 227 in the diet during days 6–16 of gestation. SP 227 (Batch no. PPC 2809) was administered at concentrations that provided dose levels of 0 (control), 0.12, 0.68 and 3.00 g/kg body weight/day. During the observation period (days 0–20 of gestation) the rats were observed for clinical signs, and body weights and food consumption were recorded. On day 20 of gestation the rats were killed and the foetuses were delivered by caesarean section. The number of corpora lutea and the number and position of all implant sites were recorded, with implant sites being categorized as live implantations, foetal deaths, late embryonic deaths or early embryonic deaths. Each foetus was weighed and examined for externally

visible abnormalities. Approximately half of the fetuses were examined for soft-tissue abnormalities by the method of Wilson (1965); the remainder were examined for gross soft-tissue abnormalities and their skeletons were stained with Alizarin Red S and examined for abnormalities and variants.

There was no effect on the dam or fetuses at any of the doses tested, including 3 g/kg/day.

Irritation studies

Skin irritation in rabbits (Stavnsbjerg, 1983 and 1984a)

Two primary skin irritation tests were carried out (one on each of Batch nos PPC 1247 and PPC 1317) following the method of the Code of Federal Regulations 1979, Title 16, No. 1500.41, adapted to OECD Guidelines for Testing of Chemicals. The backs of six albino rabbits were clipped free of hair and 0.5 g of SP 227 was introduced under gauze (1 in. $\times$ 1 in.). Test sites were divided into abraded and intact skin. Patches were secured for the 4-hr exposure period and covered by impervious material. After 4 hr, patches and the remaining SP 227 were removed with water and any skin reactions were evaluated about 1, 24, 48 and 72 hr after removal. A primary irritation index (PII) was calculated and was used to classify SP 227 as follows: PII = 0, non-irritant; $0 < \text{PII} \leq 2$, mild irritant; $2 < \text{PII} \leq 5$, moderate irritant; $\text{PII} > 5$, severe irritant.

SP 227, Batch 1247 had a PII of 0.06 and was thus classified as a mild irritant to skin; SP 227, Batch 1317 had a PII of 0 and was classified as non-irritant to skin. The mild irritant category for Batch 1247 resulted from three rabbits having very slight oedema at the dosing site about 1 hr after patch removal.

Eye irritation in rabbits (Agger, 1982d; Stavnsbjerg, 1984b)

Two eye irritation tests were carried out (one on Batch PPC 1247 and one on Batch PPC 1317) following the OECD Guidelines for Testing of Chemicals with evaluations made following the Code of Federal Regulations, Title 16, No. 1500.42. Six albino rabbits were used; the equivalent of about 0.1 ml SP 227 was instilled into the conjunctival sac of the left eye and the grade of ocular reaction was recorded at 1, 24, 48 and 72 hr after instillation. Neither batch of SP 227 produced any corneal or iris reactions at any time point; with Batch PPC 1317 there was also no conjunctival reaction and this batch was regarded as negative in its potential for eye injury. However, Batch PPC 1247 produced conjunctival reddening in four out of six rabbits after 1 hr, and the reddening was still present in one of the rabbits after an extended observation period of 7 days. As a result, this batch was regarded as positive in its potential for eye injury.

Skin sensitization: delayed contact hypersensitivity in guinea-pigs (Cuthbert and D'Arcy-Burt, 1984)

The study was performed after the method described by Buehler (1965). SP 227 (Batch no. 1317) and dinitrochlorobenzene (DNCB, an established sensitizer), were tested for induction and challenge

procedures on groups of 20 guinea-pigs each. A closed patch with 0.5 ml test solution was applied to the clipped back of the guinea-pigs for 6 hr on each of three occasions, test solutions for SP 227 being prepared as 50% (w/w) in distilled water and for DNCB as 0.2% (w/w) in propylene glycol. 2 wk after the third application the animals were rechallenged with identically prepared patches. Two further groups, each of 20 previously untreated guinea-pigs, were also exposed simultaneously with the rechallenge using identical patches, in order to check for irritancy. The incidence and severity of the reactions were examined 24 and 48 hr after final patch removal; health status was monitored throughout the study.

In both the SP 227 rechallenge and SP 227 irritancy groups negative results were recorded. In the DNCB irritancy group there were negative results, and in the DNCB rechallenge group 15/20 guinea-pigs showed evidence of sensitization. It was concluded that sensitization had not been shown with SP 227.

Mutagenicity

Gene mutation (Pedersen, 1984)

SP 227 (Batch no. PPC 1317) was examined for mutagenic activity using *Salmonella typhimurium* strains TA1535, TA100, TA1537 and TA98.

Since the test material was a crude enzyme preparation containing free amino acids such as histidine, a liquid culture assay was used. Bacteria were exposed to five doses of SP 227 (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 of viable cells were estimated.

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

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

No dose-related increases in revertants to prototrophy were obtained in any of the tests performed. All results were confirmed in an independent experiment. It was concluded that there were no indications of mutagenic activity of SP 227 in the presence or absence of metabolic activation.

Chromosome aberrations (Asquith, 1983)

Groups of five male and five female Sprague-Dawley rats were dosed orally by gavage for five consecutive days with 0, 0.3, 1.0 or 3.0 g SP 227 (Batch no. PPC 1317) kg body weight/day and killed 6 hr after the final dose. A further group of five male and five female rats was given a single ip dose of 100 mg methyl methanesulphonate/kg as a positive control and these rats were killed after 24 hr. One hour before the rats were killed they were treated with demecolcine to arrest cells in metaphase. Slides of bone marrow cells were prepared and examined

microscopically for chromosomal damage and mitotic indices were derived.

The positive control rats showed clear chromosome damage. There were no significant increases in chromosome aberrations, excluding gaps, in males or females at any dose level of SP 227. Female rats treated with SP 227 showed increases in aberrations including gaps at all doses but with no clear dose relationship and only statistically significant (chi squared = 5.02; $P = 0.025$) at 3.0 g/kg/day. Treatment with SP 227 had no effect on the mitotic capacity of bone marrow cells and it was concluded that SP 227 was not a chromosome mutagen for the rat *in vivo*.

Ecotoxicology

These ecotoxicology studies were carried out (Lindgaard-Jorgensen and Ole Kusk, 1983) on SP 227 (Batch no. PPC 1317). In the first, concentrations of SP 227 were tested for their ability to immobilize the water flea *Daphnia magna* over a 48-hr period under static conditions (Method ISO/DIS/6341). Five *Daphnia* were placed in a test-tube containing an aqueous solution of SP 227 at 0 (control), 0.1, 0.2, 0.3, 0.6 or 1.0 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.

The results from the positive controls indicated a 24-hr immobilization (initial) concentration (IC(I)₅₀) value of 1.09 mg potassium dichromate/litre, which is within the published ISO/2/interlaboratory value range (0.9 to 1.5 mg/litre; Water Quality Institute, 1979), confirming that these *Daphnia* were appropriately sensitive to a positive control. IC(I)₅₀ values for 24 and 48 hr were >1 g/litre and about 1 g/litre, respectively, for SP 227. At 0.6 g/litre no immobilized *Daphnia* were observed. Thus SP 227 was designated as practically non-toxic/non-hazardous to *Daphnia*.

A 96-hr semi-static test for acute toxicity in zebra fish (Method ISO/DIS/7346) was also carried out. Five zebra fish (*Brachydanio rerio*) were maintained in an aquarium containing an aqueous solution of SP 227 (Batch no. 1317) at 0, 0.1, 1.0 or 10 g/litre. Two such aquaria were used at each concentration. Solutions were renewed every 24 hr. An equivalent study was carried out using concentrations of potassium dichromate to provide a positive control.

The results from the positive control indicated a 24-hr lethal (initial) concentration (LC(I)₅₀) value of 240 mg/litre, which is within the published ISO/2/interlaboratory value range (200 to 400 mg/litre; Water Quality Institute, 1979) confirming that these zebra fish were sensitive to the positive control. All fish in the 10.0 G SP 227/litre group died within 20 hr of initial exposure, but all other fish survived. The death of the 10.0 g/litre group was ascribed to oxygen depletion, attributable to SP 227. From the results of this study, SP 227 was designated as non-hazardous to zebra fish.

A biodegradability test (including toxicity test to micro-organisms) with recording of biochemical oxygen demand (BOD) and total organic carbon (TOC) was performed, according to the UK version

of the Japanese MITI test (OECD Guidelines Test No. 301C). SP 227 Batch no. PPC (1317) was used in this test, and the inoculum used was freshly collected activated sludge from domestic sewage suspended in an inorganic salt medium. Sealed flasks were maintained in the dark at 25°C for 21 days with continuous stirring. Nine flasks were used, as follows: (i) inoculated medium with 100 mg SP 227/litre (duplicate flasks); (ii) inoculated medium only (duplicate flasks); (iii) inoculated medium with 100 mg sodium benzoate/litre (inoculum activity test with reference compound); (iv) inoculated medium with 100 mg SP 227/litre and 100 mg sodium benzoate/litre (duplicate flasks)—the toxicity test; (v) medium with 100 mg SP 227/litre and without sludge inoculation (duplicate flasks)—the physical/chemical control. Oxygen consumption in each flask was measured with respect to time.

The test was concluded on day 21 because complete degradation had occurred after 14 days. The oxygen uptake curve of the flasks containing SP 227 and sodium benzoate together showed no inhibition of sodium benzoate degradation, and the test was regarded as valid since the criteria of 60% removal of theoretical oxygen demand and 60% removal of dissolved organic carbon were exceeded during the biodegradation of sodium benzoate.

Since oxygen uptake and dissolved organic carbon removal both showed 78% biodegradation of SP 227 during the test period, SP 227 was designated as readily biodegradable.

Pathogenicity of *Humicola insolens* 9510

The potential pathogenicity of the alkaline cellulase-producing micro-organism *Humicola insolens* was investigated in mice and rabbits after parenteral injection in order to optimize the opportunity for systemic infection (Aarup, 1982a,b).

Four groups of six NMRI mice were dosed ip once with 10¹, 10³, 10⁴ or 10⁵ colonies of *H. insolens* 9510/kg body weight. A further four groups of mice were given the same doses 2 days after they had been given a sc dose of 5 mg cortisone to act as an immunosuppressant. Two further groups (one with and one without cortisone pretreatment) were given sodium chloride solution and acted as controls.

There were no treatment-related deaths. After 20 days survivors were autopsied. The majority of immunosuppressed mice that had been treated with 10³, 10⁴ or 10⁵ colonies/kg showed scattered pus-filled abscesses on the liver and omentum. In normal mice the same findings were observed, but to a much lesser extent, in all mice treated with 10⁵ colonies/kg and in one animal treated with 10³ colonies/kg. Since the LD₅₀ value was >10⁵ colonies/kg it was concluded that *H. insolens* was avirulent to mice.

Three Dutch Landstrain rabbits were shaved on the back, and a grid of 16 dosing sites was intracutaneously dosed on one occasion with 10⁻¹, 10¹, 10² or 10³ colonies/site or with sodium chloride solution. The rabbits were observed for 15 days and local skin reactions were measured on four occasions over this period. At the end of 15 days the rabbits were autopsied. There were no signs of systemic infection, but the sites intracutaneously dosed with 10¹, 10² or

10³ colonies developed minor inflammatory lesions. By day 6 after dosing these had developed into superficial, localized abscesses which enucleated on day 9. By the end of the study the inflammation had almost healed and only a little fibrosis was present. It was concluded that *H. insolens* in a dose of 10 colonies/site and above provoked development of small, localized, superficial abscesses, which could represent either some pathogenic ability or a foreign body reaction. In any case the reactions were mild.

Discussion

A previous publication (Hjortkjaer *et al.*, 1986) indicates that the safety evaluation of microbial enzymes should be based on knowledge of the source micro-organism and toxicological testing of the enzyme preparation in animal models. No-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 TOS residues in final products and data on human consumption. Human exposure to alkaline cellulase is either by ingestion of foodstuffs prepared using it, or by exposure during its production process.

It has been estimated that in Denmark mean beer consumption is 341 g/person/day (Emborg, 1983) and that since beer contains approximately 0.0044 g enzyme TOS/litre a 60-kg man would consume 0.025 mg enzyme TOS/kg/day. Similarly, it has been estimated that 111 g bread is eaten daily per head of population, and it can therefore be calculated that enzyme TOS consumption in bread amounts to approximately 0.097 mg/kg. The total daily consumption through bread and beer is therefore in the region of 0.1 mg enzyme TOS/kg/day. This compares with the demonstrated no-adverse-effect level in rats of at least 420 mg enzyme TOS/kg, giving a safety margin in excess of 4000. This, together with the absence of any adverse effects in teratogenicity and mutagenicity studies and the history of cellulase use in food production make it possible to conclude that alkaline cellulase can be regarded as safe for the consumer in the applications given. Furthermore, the ready biodegradability and absence of hazard to *Daphnia* and zebra fish indicates that alkaline cellulase is a material of low environmental impact.

These data indicate that *H. insolens* can be regarded as non-toxicogenic and non-pathogenic. Inhalation, irritation and sensitization studies indicate that no special occupational health preparations need be applied in the manufacture and use of alkaline cellulase.

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