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Safety Evaluation of Amino Peptidase Enzyme Preparation Derived from *Aspergillus niger*

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Abstract—An amino peptidase enzyme preparation obtained from *Aspergillus niger* was subjected to a series of toxicological tests to document the safety for use as a processing aid for food. The enzyme preparation was examined for subacute and subchronic oral toxicity, and mutagenic potential. No evidence of oral toxicity or mutagenicity was found. Administration of the amino peptidase enzyme preparation at doses of 500, 1000 and 2000 mg/kg body weight/day for 90 days did not induce noticeable signs of toxicity. The no-observed-adverse-effect level (NOAEL) of the enzyme preparation in the subchronic toxicity study was 2000 mg/kg body weight/day (equivalent to 1152 PHEA units/kg body weight/day). It can be concluded that no safety concerns were identified in the studies conducted with this amino peptidase enzyme preparation derived from *Aspergillus niger* and produced under controlled fermentation conditions.

Keywords: *Aspergillus niger*; amino peptidase; safety; toxicology.

Abbreviations: PHEA units = phenyl alanine units; TOS = total organic solids; OECD = Organisation for Economic Cooperation and Development; NOAEL = no-observed-adverse-effect level; EDI = estimated daily intake; BW = body weight.

INTRODUCTION

Enzymes from *Aspergillus niger* have already been used in food production for several decades (AMFEP, 1997). Protease enzymes derived from *A. niger* normally contain a range of different peptidases to enable the organism to degrade the various proteins found in plants. Experiments have shown that one of these peptidases, belonging to the group of the so-called amino peptidases, can play an important role in the preparation of bread and cheese. Therefore, an *A. niger* strain has been selected which effectively produces this specific amino peptidase.

The amino peptidase is able to generate specific free amino acids from the proteins present in dough and (semi-) hard cheeses. These free amino acids are normal constituents of the diet. In the dough, the amino acids are subsequently converted by the baker's yeast into alcohols and esters which are also normal constituents of the diet. By generating free amino acids, the addition of the amino peptidase preparation results in an improvement of the flavour and aroma of baked products and cheese.

The safety of microbial food enzymes can be established on the basis of a history of safe use of the production organism, supplemented with scientific studies of the enzyme preparation (Battershill, 1993; SCF, 1992).

The production organism of amino peptidase is *A. niger*. This is generally considered as a safe, non-pathogenic, fungus to humans, animals and plants. The fungus is known to occur naturally in foods. It is commonly present in products such as rice, seeds, nuts, olives and dried fruits. For several decades *A. niger* has been used safely in the commercial production of organic acids and various food enzymes, such as glucose oxidase, pectinase, α-amylase and amyloglucosidase. Industrial production of citric acid by *A. niger* has taken place since 1919. In the US, enzyme preparations from *A. niger* have had a 'GRAS (Generally Recognized as Safe) pending' status since 1973.

Although products from *A. niger* have been used for many decades, there is no evidence that the

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industrial strains used are able to produce toxins. The non-toxicogenicity of *A. niger* enzyme preparations has been confirmed by a large number of toxicological tests, as well as testing batches of the various end-products for toxins. These toxicological studies performed on various enzyme preparations from *A. niger* provide a basis for a safety evaluation by the Joint Expert Committee on Food Additives (JECFA) of the FAO/WHO (FAO/WHO, 1975, 1987 and 1990). Although not justified by the results of the toxicological studies, JECFA first allocated a numerical acceptable daily intake (ADI) to enzyme preparations of *A. niger*, based on the fear that some strains may produce unknown toxins (FAO/WHO, 1987). However, the long history of use of *A. niger* as a food enzyme source, the substantial amount of safety studies, as well as other scientific reports submitted to JECFA showed that toxin production is very unlikely. Therefore, JECFA reconsidered its decision in 1990 and changed the ADI for enzyme preparations derived from *A. niger* to "not specified" (FAO/WHO, 1990). Apart from a positive evaluation of JECFA, most countries that regulate the use of enzymes, such as the US, France, Denmark, Australia and Canada, have accepted the use of enzymes from *A. niger* in a number of food applications.

Table 1. Analytical results of the amino peptidase tox-batch (Snuverink, 1998)

Parameter	Unit	Batch A	Batch B	Tox-batch
Identity:				
Appearance		lump free powder	lump free powder	lump free powder
Colour		creamish	creamish	creamish
Odour		typical	typical	typical
Identification (SDS–PAGE)		conforms	conforms	conforms
Foreign matter		absent by test	absent by test	absent by test
Enzymatic assay				
Amino peptidase	PHEA/g	570	690	576 (600@)
Protease	PCU/g	162	< 800†	nd
Material balance				
Dry matter	%	87.2	89.5	87.3
Ashes	%	26.4	23.9	28.2
Total protein (Kjeldahl × 6.25)	%	15.9	18.5	nd
Total carbohydrates	%	6.68	6.59	nd
Total organic solids (TOS‡)	%	22.6	23.5	21.7
Antifoam	%	< 0.1	< 0.1	nd
Residual minerals				
Heavy metals (as Pb)	mg/kg	< 30	< 30	nd
Pb	mg/kg	0.14	0.2	nd
Cd	mg/kg	0.04	0.05	nd
As	mg/kg	0.20	< 0.07	nd
Hg	mg/kg	< 0.04	< 0.04	nd
K	mg/kg	3750	3540	nd
Na	mg/kg	3430	2310	nd
Ca	mg/kg	2870	2350	nd
Mg (calculated)	mg/kg	90000	92800	(91400)
Stability 21°C during 48 hr	%	nd	nd	> 90
Stability 4°C during 156 hr	%	nd	nd	> 90
Toxins				
Aflatoxin B_1	μg/kg	< 10	< 10	nd
T2 toxin	μg/kg	< 100	< 100	nd
Ochratoxin A	μg/kg	< 40	< 40	nd
Zearalenone	μg/kg	< 200	< 200	nd
Antimicrobial activity		absent by test	absent by test	nd
Microbiological determinations				
Standard plate count	CFU/g	< 10	400	730
Salmonella	/25 g	not detectable	not detectable	nd
Coliforms	CFU/g	< 3	< 3	nd
Escherichia coli	/25 g	not detectable	not detectable	nd
Staphylococcus aureus	/g	not detectable	not detectable	nd
Moulds	CFU/g	30	< 10	nd
Yeasts	CFU/g	< 10	< 10	nd
Production strain	/25 g	not detectable	not detectable	nd

One amino peptidase-unit (PHEA) is defined as the amount of enzyme which hydrolyses 1 micromol *p*-nitroanilide per minute from phenylalanine-*p*-nitroanilide at 30°C and pH 7.2 under the conditions described of the test.
†Detection limit for dilution used.
‡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 21.7% in the tox batch,
nd = not determined;/CFU = colony forming units; @ activity at end of tox studies.

Although the above data indicate that the chance that the amino peptidase production strain adds any risks to the final product is very small, an extensive testing programme has been performed to confirm the safety of amino peptidase from this *A. niger* strain.

This paper describes studies conducted to examine the amino peptidase enzyme preparation for subacute and subchronic toxicity as well as mutagenic potential in detail.

MATERIALS AND METHODS

The batch of amino peptidase used for toxicity testing (referred to as the "tox-batch") was a mixture of samples from two separate fermentations that were produced by the procedure used for the commercial preparation of amino peptidase. 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 two, non-standardized samples. The two powdered samples were mixed (1/1, w/w), analysed and used as tox-batch. In Table 1, the results of the characterization of the two samples and the tox-batch by chemical and microbial analysis are presented (Snuverink, 1998). 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 576 PHEA units/g with a TOS [Total Organic Solids = 100%–water (%)–ash (%)–diluent (%)] value of 21.7%. As 1 g of enzyme preparation corresponds to 576 PHEA units, 1 PHEA unit corresponds to 1000*0.217/576 = 0.377 mg TOS).

All studies were carried out in accordance with current guidelines of the Organisation for Economic Cooperation and Development (OECD, 1984), and in compliance with the principles of Good Laboratory Practices (GLP), according to OECD principles of GLP, May 1981. The studies were carried out at Huntingdon Life Sciences, Eye, Suffolk, UK, during the period November 1996 to September 1997:

Subacute 14-day oral toxicity in rat;
Subchronic 90-day oral toxicity in rat;
Bacterial gene mutation, Ames test;
In vitro chromosomal aberration test, human lymphocytes.

Oral toxicity studies

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 CD (Crl:CD® (SD)BR) rats, which received the tox-batch in purified water, by oral gavage, at doses of 0 (control), 500, 1000 and 2000 mg/kg body weight/day. All formulations were prepared freshly each day. Food and water were available *ad lib*. Routine clinical observations, body weight and food consumption were measured throughout the study periods. At day 14, animals were necropsied and macroscopic observation and organ weights (Table 2) were recorded. Several tissues (Table 2) were collected and preserved for possible future histopathological examination (Jackson, 1997a).

Subchronic 90-day oral toxicity in rats

In a subchronic 90-day toxicity study (OECD, 1984), the tox-batch was administered to SPF-bred CD rats by oral gavage. The study consisted of four groups, each comprising 10 males and 10 females. The formulations were prepared as a series of graded concentrations in purified water to provide the required doses at a constant volume-dosage of 10 ml/kg body weight. Based on the 14-day range-finding study the doses 0, 500, 1000 and 2000 mg/kg body weight/day were selected.

Routine clinical observations, body weight, food consumption and ophthalomoscopy were measured throughout the study periods. During week 13, blood was collected from each animal for clinical laboratory investigations. At the end of week 13, all animals were necropsied and macroscopic observations were recorded. The weights of organs as listed in Table 2 were measured at autopsy and organ/body weight ratios were calculated. Tissues (Table 2) collected from all animals of the control and highest dose group, as well as all gross lesions of all animals (all dose groups) were processed and slides were examined (Jackson, 1997b).

Mutagenicity

Ames test

The tox-batch was examined for its mutagenic potency in four histidine-requiring *Salmonella typhimurium* mutant strains TA98, TA100, TA1535 and TA1537 and one tryptophan-requiring *Escherichia coli* mutant strain CM891, using treat-and-plate assays (Ames *et al.*, 1975; Maron and Ames 1983; OECD 1984). Tester bacteria were exposed to five concentrations ranging from 0.1 to 10 mg/ml in phosphate buffered nutrient broth both in the absence and presence of a rat liver-derived metabolic activation system (S-9 mix). After incubation, the exposed cells were separated from the tox-batch by centrifugation prior to plating. Negative and positive controls were run simultaneously with the test (May, 1997).

In vitro chromosomal aberration test with human lymphocytes

The tox-batch was examined for its potential to induce structural chromosome aberrations in cultured human peripheral lymphocytes following the

Table 2. Organs and tissues weighed, preserved and microscopically examined in the 14- and 90-day oral toxicity study with amino peptidase tox-batch

	14-day study		90-day study		
Organ/tissue	Weighed	Fixed	Weighed	Fixed	Light microscopy
Abnormalities	X	X	X	X	X
Adrenals	X	X	X	X	X
Aorta–thoracic				X	X
Brain	X	X	X	X	X
Caecum		X		X	X
Colon		X		X	X
Duodenum		X		X	X
Epididymides	X	X	X	X	X
Eyes				X	X
Femur				X	X
Heart	X	X	X	X	X
Ileum		X		X	X
Jejunum		X		X	X
Kidneys	X	X	X	X	X
Lachrymal glands				X	X
Liver	X	X	X	X	X
Lungs and bronchi	X	X	X	X	X
Lymph nodes –mandibular		X		X	X
–mesenteric		X		X	X
Mammary gland-caudal/cranial				X	X
Marrow smear				X	
Oesophagus		X		X	X
Ovaries	X	X	X	X	X
Pancreas				X	X
Pituitary				X	X
Prostate		X		X	X
Rectum		X		X	X
Salivary glands–submandibular				X	X
Sciatic nerves		X		X	X
Seminal vesicles				X	X
Skeletal muscle–thigh				X	X
Skin				X	X
Spinal cord		X		X	X
Spleen	X	X	X	X	X
Sternum				X	X
Stomach		X		X	X
Testes	X	X	X	X	X
Thymus	X	X	X	X	X
Thyroid with parathyroids	X	X	X	X	X
Tongue				X	X
Trachea		X		X	X
Urinary bladder		X		X	X
Uterus with cervix	X	X	X	X	X
Vagina				X	X

Examined if results suspected during study.

methods of OECD (1984). The test was conducted with and without the inclusion of a rat liver-derived metabolic activation system (S-9 mix). In the absence of S-9 mix, the cells were exposed continuously for 19 and 43 hr to the tox-batch; in the presence of S-9 mix the cells were exposed for 3 hr to the tox-batch, washed and cultured for an additional 16 or 40 hr.

At the 19-hr sampling time, three concentrations of the tox-batch (in the absence of S-9 mix: 625, 2500 and 5000 μg/ml and in the presence of S-9 mix: 1250, 2500 and 5000 μg/ml), together with the

Table 3. 14-Day oral toxicity study with amino peptidase tox-batch: results of BW, FC, FCE and relative lung weights

Group/sex	Enzyme dose (mg/kg bw/day)	Total food consumption g/animal (% of control)	Total body weight gain, g (% of control)	Food conversion efficiency (group mean values, %)	Lung weight relative to body weight
1 M[b]	0	321 (100)	99 (100)	30.8	0.585 ± 0.050
2 M	500	338 (105)	98 (99)	29.0	0.639 ± 0.026
3 M	1000	322 (100)	105 (106)	32.6	0.642 ± 0.097
4 M	2000	336 (105)	100 (101)	29.8	$0.832^{a} \pm 0.122$
1F	0	272 (100)	55 (100)	20.2	0.800 ± 0.226
2F	500	250 (92)	50 (91)	20.0	0.703 ± 0.052
3F	1000	243 (89)	48 (87)	19.8	0.837 ± 0.186
4F	2000	232 (85)	48 (87)	20.7	0.690 ± 0.025

[a] $P < 0.05$ Behren's Fisher Test.
[b] n = five animals per group.

Table 4. 90-Day oral toxicity study with amino peptidase tox-batch: results of BW, FC, FCE and kidney effects

Group/sex	Enzyme dose (mg/kg bw/day)	Total food consumption g/animal (% of control)	Total body weight gain, g (% of control)	Food conversion efficiency (group mean values, %)	Cortico-medullary mineralization kidney Score: minimal; slight; moderate
1 M[b]	0	2463 (100)	342 (100)	13.9	—
2 M	500	2379 (97)	330 (96)	13.9	—
3 M	1000	2636 (107)	364 (106)	13.8	—
4 M	2000	2417 (98)	324 (95)	13.4	—
1F	0	1820 (100)	141 (100)	7.7	5/10; 3/10; 0/10
2F	500	1863 (102)	144 (102)	7.7	0/1; 0/1; 1/1
3F	1000	1746 (96)	133 (94)	7.6	1/1; 0/1; 0/1
4F	2000	1806 (99)	136 (96)	7.5	2/10[a]; 0/10; 0/10

[a] $P < 0.05$; two-tailed Fisher's exact probability test.
[b] n = 10 animals per group; for kidney mineralization only one female of intermediate doses examined.

negative (vehicle) and positive control were selected for the analysis of chromosomal aberrations. At the 43-hr sampling time, in both the absence and presence of S-9 mix one concentration of the tox-batch (5000 μg/ml) together with the negative (vehicle) and positive control were selected for the analysis of chromosomal aberrations. 100 metaphases were analysed from all selected cultures, except for cultures treated with cyclophosphamide at 12 μg/ml for 43 hr in the presence of S-9 mix, for which a total of only 155 metaphases could be located from two cultures (Strang, 1997).

RESULTS

Subacute 14-day oral toxicity (range-finding) study in rats

There were no deaths or signs to reaction of treatment. Body weight gain and food consumption were slightly low for treated females in comparison with the control, but the food conversion efficiency was unaffected by treatment, indicating that the inferior body weight gain was entirely due to the reduced food consumption and was, therefore, considered to be of no toxicological significance

Table 5. Subchronic 90-day oral toxicity study of amino peptidase tox-batch in rats: relevant haematological and biochemical results

Blood parameters	Treatment levels							
Dose levels (mg/kg/day tox-batch)	Group 1 M control	Group 2 M 500 mg/kg	Group 3 M 1000 mg/kg	Group 4 M 2000 mg/kg	Group 1F control	Group 2F 500 mg/kg	Groups 3F 1000 mg/kg	Group 4F 2000 mg/kg
PCV	0.44 d	0.44	0.43	0.45	0.42	0.42	0.42	0.41
	0.02	0.03	0.02	0.02	0.01	0.01	0.01	0.03
HB	15.2	15.5	14.9	15.6	15.0	15.1	15.0	14.5
g/dl	0.7	0.9	0.7	0.8	0.6	0.3	0.4	1.0
RBC	8.38	8.46	8.25	8.64	7.83	7.89	7.75	7.70
10**12	0.38	0.62	0.33	0.49	0.30	0.19	0.30	0.65
Total WBC	11.8	10.9	10.2	11.2	8.6	7.6	7.8	7.3
10**9	2.3	3.1	2.6	2.7	3.7	1.8	3.8	3.8
PLAT	1083	1057	1061	1169	1033	1068	1074	1127
10**9	131	126	122	156	91	103	116	192
PT	13.3	13.1	12.5	13.0	11.9	12.3[a]	12.5[c]	12.6[c]
sec	1.5	1.0	0.7	1.4	0.5	0.3	0.3	0.4
ALP	86	85	90	89	54	55	65	57
IU/litre	14	17	24	17	9	19	21	22
ALT	46	41	38[a]	39[a]	42	37	36	30
IU/litre	10	7	6	4	13	11	11	7
GGT	0	0	0	0[a]	0	0	0	1
IU/litre	0	0	0	1	1	1	0	1
BILT	1	1	2	1	2	2	2	3
μmol/litre	1	0	1	1	1	0	1	1
CHOL	1.9	1.8	1.8	1.9	2.5	2.2	2.1[a]	2.3
mmol/litre	0.2	0.3	0.4	0.3	0.4	0.3	0.4	0.4
TRIG	0.88	0.98	0.99	1.14[a]	0.81	0.97	0.76	0.76
mmol/litre	0.21	0.25	0.35	0.28	0.33	0.38	0.28	0.27
TP	62	62	60	63	69	70	67	66
g/litre	3	2	3	3	7	5	2	3
Phos	1.96	2.02	2.09	2.21[b]	1.58	1.69	1.48	1.62
mmol/litre	0.18	0.12	0.12	0.20	0.31	0.36	0.21	0.29

a,b,c: Student's *t*-test based on pooled error variance significance. a: $P < 0.05$; b: $P < 0.01$ and c: $P < 0.001$; d: Values are mean $\pm$ SD of n = 10 rats.

Parameters haematology: packed cell volume (PCV), haemoglobin (HB), red blood cells (RBC), total leucocyte count (WBC), platelets (PLAT) and prothrombin time (PT); Parameters biochemistry: alkaline phosphatase (ALP), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (GGT), total bilirubin (BILT), total cholesterol (CHOL), triglycerides (TRIG), total protein (TP) and inorganic phosphorus (Phos).

Data not shown from blood parameters: mean cell haemoglobin concentration (MCHC), mean cell volume (MCV), mean cell haemoglobin (MCH), differential leucocyte count; aspartate aminotransferase; creatinine; urea; glucose; albumin, albumin/globulin ratio and electrolytes.

Table 6. Bacterial mutagenicity assays with amino peptidase tox-batch

		Mean revertant colonies/plate with strain									
		TA98		TA100		TA1535		TA1537		CM891	
Tox-batch treatment	Addition (mg/ml)	−S-9	+S-9[a]	−S-9	+S-9	−S-9	+S-9	−S-9	+S-9	−S-9	+S-9
			first assay								
Tox-batch	10	30[b]	28	92	81	21	18	12	8	168	156
Tox-batch	3.16	30	29	90	91	20	21	11	12	157	153
Tox-batch	1	27	31	99	86	18	19	14	11	174	161
Tox-batch	0.32	31	30	90	91	21	21	12	9	150	160
Tox-batch	0.1	29	33	92	93	21	19	13	9	174	154
Purified water	0	32	33	100	98	23	24	15	14	163	155
Positive control	c	1145	119	1415	986	428	121	2540	111	1441	982
			second assay								
Tox-batch	10	33	25	97	98	21	23	16	15	193	151
Tox-batch	3.16	32	30	101	99	20	21	14	14	184	165
Tox-batch	1	27	29	104	104	21	21	17	14	179	161
Tox-batch	0.32	32	32	93	105	20	19	15	13	191	167
Tox-batch	0.1	25	32	94	102	21	20	18	17	146	166
Purified water	0	29	33	99	98	20	21	18	18	170	183
Positive control	c	824	118	1443	1063	687	111	1948	112	909	1609

[a]The S-9 mix was checked for sterility and found sterile.
[b]Each experiment was carried out using triplicate plates.
[c]Positive controls: *N*-ethyl-*N*′-nitro-*N*-nitrosoguanidine: 10 μg with strain TA100 −S-9, TA1535 −S-9 and ENNG 5 μg CM891 −S-9
Benzo[*a*]pyrene: 5 μg with strain TA98 +S-9, TA1537 +S-9
9-Aminoacridine: 2 μg with strain TA1537 −S-9
2-Nitrofluorene: 20.0 μg with strain TA98−S-9
2-Aminoanthracene: 5.0 μg with strain TA100 +S-9, TA1535 +S-9 and CM891 +S-9.

(Table 3). Although the group mean absolute and body weight–relative lung weights for males receiving 2000 mg/kg body weight/day were high, this was due to two animals which had incomplete collapse of the lungs at macroscopic examination (Table 3). This finding is not uncommon in young rats and is considered to be related to the use of CO_2 euthanasia; the distribution is considered to be fortuitous. This was confirmed by the absence of lung changes in the 90-day oral toxicity study.

It was concluded that administration of the tox-batch to CD rats at doses up to 2000 mg/kg body weight/day did not result in any changes which were considered, at this stage, to be of toxicological significance (Jackson, 1997a).

Subchronic 90-day oral toxicity in rats

Accuracies of preparation and homogeneity of the tox-batch in the vehicle were demonstrated by analyses of the protein content. Stability of the tox-batch in vehicle was demonstrated by analysis of the amino peptidase enzyme activity. In Table 4, the mean data of food consumption, food conversion efficiency, body weight gain, enzyme dose and kidney effects are given. No differences among overall body weight gain, food consumption, food conversion efficiency and organ examination that were considered to be an effect of treatment, were noticed. Moreover, ophthalmoscopic examinations did not reveal any treatment-related effect.

Prothrombin times were slightly longer for treated females than for controls (Table 5). As, the values were within background levels seen at these laboratories and a similar effect was not present in males, the values were considered to be due to a normal biological variation. Other haematology values, namely white blood cell count, red blood cell count, haemoglobin, haematocrit and platelet count (Table 5) and leucocyte differentials from treated animals did not differ from controls.

A slightly dose-related decrease in plasma alanine aminotransferase values was recorded for both males and females. Reductions in circulating levels of hepatic enzymes have no known toxicological significance and the finding is therefore considered incidental. Males receiving 2000 mg/kg/day revealed slightly high triglyceride and phosphorous levels in comparison with controls. These values were within background levels observed at these laboratories and in the absence of a dose relationship and a similar effect in the females, were not considered of toxicological significance. There were no differences related to the tox-batch among groups for blood chemistry values [alkaline phosphatase, γ-glutamine transpeptidase, total bilirubin, cholesterol, total protein (Table 5), aspartate aminotransferase, blood urea, creatine, glucose, albumin, albumin/globulin ratio and electrolytes (results not shown)]. No treatment-related changes in absolute and relative organ weights as listed in Table 2 were recorded. Histopathological examination reveal a decreased incidence and severity of cortico-medullary mineralization in the kidney of females receiving 2000 mg/kg body weight/day (Table 4). There were no other microscopic changes that were considered to be an effect of treatment.

It was concluded that administration of the tox-batch at doses of 500, 1000 and 2000 mg/kg body weight/day did not induce noticeable signs of toxicity. The no-observed-adverse-effect level (NOAEL) of the tox-batch in the subchronic study was therefore 2000 mg/kg body weight/day (Jackson, 1997b).

Mutagenicity—Ames test

In order to overcome growth enhancement and possible artefactual test results due to the proteinaceous nature of the tox-batch in the *in vitro* bac-

Table 7. *In vitro* human lymphocyte assay with amino peptidase tox-batch: results

Test details	Treatment (μg/ml)	Reduction in mean mitotic index (%)[a]	Mean (%) cells with aberrations (including gaps)	Cells with aberrations (excluding gaps)
19-hr sampling time –S-9	0	—	3.0	1.0
	625	increase	5.0	1.5
	2500	9	5.0	2.0
	5000	23	6.5	2.0
	mitomycin C (0.1)	—	36.0***	24.0***
19-hr sampling time +S-9	0	—	6.0	1.5
	1250	0	5.0	2.0
	2500	increase	6.5	3.0
	5000	increase	4.5	1.5
	cyclophosphamide (6)	—	26.0***	21.5***
43-hr sampling time –S-9	0	—	3.5	1.0
	5000	increase	4.5	1.0
	mitomycin C (0.1)	—	45.0***	38.5***
43-hr sampling time +S-9	0	—	4.5	0.5
	5000	increase	8.5	2.5
	cyclophosphamide (12)	—	47.7***	40.0***

***Very highly significant, $P < 0.001$ of increase in frequency of aberrant metaphases in treated cultures, compared with negative control values.

[a]Reduction in mean mitotic index compared with negative control values.

terial gene mutation test which could lead to false positive test results (Verhagen *et al.*, 1994), it was decided to conduct a plate-and-treat assay. No increases in the number of revertants were obtained in any of the five bacterial strains at the concentrations tested, either in the presence or absence of S-9 mix. The positive control substances, benzo[*a*]-pyrene, 2-nitrofluorene, 2-aminoanthracene, 9-aminoacridine and *N*-ethyl-*N*′-nitro-*N*-nitrosoguanidine gave the expected strong increase in the number of revertants. The results are presented in Table 6, and it is concluded that the tox-batch did not show mutagenic activity under the conditions of the test (May, 1997).

In vitro chromosomal aberration test with human lymphocytes

No biologically or statistically significant increases in the frequency of metaphases with aberrant chromosomes, compared with solvent control values, were seen in cultures treated with the tox-batch, including or excluding gap-type aberrations, at both sampling times ($P > 0.05$), both in the absence and presence of S-9 mix. The results for the concentrations that were scored in this study are summarized in Table 7. The known clastogens mitomycin C (direct-acting clastogen) and cyclophosphamide (requires metabolic activation to achieve optimum activity) induced significant increases in the frequency of metaphases with aberrant chromosomes, compared with the solvent control values, at both sampling times ($P < 0.001$ in all cases), thus demonstrating the sensitivity of the procedure and the metabolic activity of the S-9 mix employed (Table 7).

It is concluded that the tox-batch did not show any evidence of clastogenic activity under the conditions of the test (Strang, 1997).

DISCUSSION AND CONCLUSIONS

The results of the 90 days oral toxicity study presented in this paper can be used to calculate the safety margin of the consumption of the amino peptidase enzyme preparation from *A. niger*.

Whereas the dosing of an enzyme preparation in its application is always based on the enzyme activity present in the preparation, the calculation of the daily intake of a preparation, as well as the safety margin, should be based on weight. The best way to express the weight is on the basis of the Total Organic Solids (TOS), as the rest of the preparation (i.e. ash, water and diluents) is fully characterized and well known as safe for consumption in the quantities involved.

For the calculation of the safety margin, it is necessary to know (a) the NOAEL of the subchronic oral toxicity study expressed as TOS, (b) the concentration of the enzyme-TOS in the final food products, and (c) the human consumption of the food products concerned.

In the subchronic oral toxicity study no adverse effects were observed at the highest dose given, that is, 2000 mg/kg body weight/day. As the percentage TOS in the enzyme preparation was 21.7%, the highest dose corresponds with 434 mg TOS/kg body weight/day.

The maximum recommended dose of the enzyme preparation in bread is 270 PHEA units/kg bread and 3 PHEA units/kg cheese. As 1 PHEA unit corresponds to 0.377 mg TOS, the maximum recommended doses in 1 kg bread and cheese thus correspond to 102 mg and 1 mg TOS, respectively.

The daily average intake of bread per person is 158 g in the UK, 123 g in Denmark and 109 g in the US (Bergman and Broadmeadow, 1997). The daily average intake of cheese in the US is 40 g (USDA, 1996). The consumption of a person with an average weight of 60 kg thus results in a maximum estimated daily intake (EDI) of 102*0.158/60 (bread) + 1*0.04/60 = 0.27 mg enzyme-TOS/kg body weight/day. The safety margin would thus be: NOAEL/EDI = 434/0.27 = 1607.

The data presented in this paper indicate that there are no reasons for safety concerns when using the amino peptidase from *A. niger* in the preparation of food.

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